Isolation and partial characterization of a protein with HMG-CoA reductase phosphatase activity associated with rat liver microsomal membranes

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Abstract Several rat liver HMG-CoA-reductase (HMG-CoA-Rd) phosphatase activities have been shown to be associated with the endoplasmic reticulum. These activities were not due to glycogen contamination, as judged not only from different patterns of solubilization of the microsomal membranes and the glycogen pellet but also by differential centrifugation behavior under standard conditions and in a sucrose gradient. We present evidence that at least three forms of protein phosphatase are associated with microsomal membranes: a polycation-stimulated type 2A phosphatase, a type 2C phosphatase, and a non-2A, non-2B, non-2C phosphatase. This last HMG-CoA-Rd phosphatase activity corresponding to an 85 kDa protein was partially purified by several chromatographic procedures. The IC_{50} value for the inhibition of the HMG-CoA-Rd phosphatase by I-2 was 10-fold higher than for the inhibition of the purified type 1 catalytic subunit from rabbit skeletal muscle. The microsomal HMG-CoA-Rd phosphatase activity was slightly affected by the protein inhibitor that inhibits type 2A activity when HMG-CoA reductase is the substrate. The HMG-CoA-Rd phosphatase activity is spontaneously active and it is not reactivated in the presence of Mg^{2+} or polycations. The holoenzyme does not contain the inhibitor-2 and it is not reactivated by incubation with ATP and glycogen synthase kinase-3. Proteolytic treatment of the enzyme yielded a polypeptide fragment of low M_r (37 kDa) with reduced activity. A model of holoenzymatic HMG-CoA-Rd phosphatase and its relation to the microsomal membranes is presented. —**Asins, G., D. Serra, and F. G. Hegardt**. Isolation and partial characterization of a protein with HMG-CoA reductase phosphatase activity associated with rat liver microsomal membranes. *J. Lipid Res.* 1991. **32:** 1391–1401.

Supplementary key words HMG-CoA reductase phosphatase • protein phosphorylation • HMG-CoA reductase • microsomes

The major regulatory enzyme in the pathway of cholesterol synthesis, HMG-CoA reductase (E.C.1.1.1.34), is anchored to both the smooth and rough endoplasmic reticulum (1). This regulatory enzyme has been shown to be regulated by covalent modification. AMP-activated HMG-CoA-reductase kinase (2, 3) inactivates and phosphorylates HMG-CoA reductase both in vitro and in vivo. Inactivated, homogenous ^{32}P-labeled HMG-CoA reductase is reactivated in vitro by rat liver cytosolic (4) and microsomal (5, 6) protein phosphatases with a concomitant release of ^{32}P bound to serine residues (7).

Four major classes of serine/threonine protein phosphatase catalytic subunits are present in the cytoplasmic compartment of eukaryotic cells (review in ref. 8). Type 1 protein phosphatase is inhibited by two heat-stable proteins called inhibitor-1 and inhibitor-2 (I-2); in contrast, type 2 phosphatases are not affected by either of these inhibitor proteins.

Protein phosphatase type 1 is the major protein phosphatase present in microsomes and the glycogen pellet in mammalian tissues. The catalytic subunit that is not in a free form, as deduced from experiments in vivo, combines with other regulatory or specific targeting subunits as a function of its subcellular location (8). In mammalian liver the catalytic subunit (37 kDa) of type 1 protein phosphatase is associated with glycogen particles through a specific G subunit (161 kDa) (9). It has been speculated that the catalytic subunit of PP-1 would bind to the hepatic microsomal membranes in a similar way because of a specific binding protein (10).

The high glycogen content usually found in microsomal preparations has brought into question the occurrence of protein phosphatases in the endoplasmic reticulum, as these phosphatases have high affinity for glycogen (11). Several authors have discussed whether or not glycogen synthase phosphatase activity is present in glycogen-

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; HMG-CoA-Rd, HMG-CoA reductase; IC_{50}, concentration causing 50% inhibition; I-2, phosphatase inhibitor-2, also called modulator; PP, protein phosphatase; PP-R, protein phosphatase associated with endoplasmic reticulum; RP-inhibitor, HMG-CoA reductase phosphatase inhibitor; PMSF, phenylmethylsulfonyl fluoride; TLCK, N-α-*p*-tosyl-L-lysine chloromethyl ketone; DTT, dithiothreitol; Bistris, 2-[bis(2-hydroxyethyl)amino]2-2(hydroxymethyl)-1,3-propanediol; PMS, postmitochondrial supernatant; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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devoid microsomes (11, 12). A recent contribution (13) shows that the microsomal glycogen synthase phosphatase activity is not explained by contamination from the glycogen-bound enzyme. The occurrence of phosphorylase phosphatase activity in microsomes has not been questioned.

The present report shows that there is an HMG-CoA-Rd phosphatase activity associated with vesicles of the endoplasmic reticulum that is not due to the contamination of glycogen, and that this phosphatase activity has three forms, which we have classified as type 2A, 2C, and a new type PP-R (protein phosphatase HMG-CoA reductase). We have partially purified this last phosphatase activity (PP-R), and we have classified it as type 1 because of its kinetic properties towards I-2 and RP-I. In addition, we present a model of interaction between the catalytic subunit of this protein phosphatase and other nonenzymatically active proteins.

MATERIALS AND METHODS

Chemicals

Most of the reagents used in this study have been reported previously (14). α-Amylase from human saliva (1,000 U/mg protein), benzamidine, calcineurine (3,000 U/mg protein), calmodulin (40,000 U/mg protein), lima bean trypsin inhibitor, liver glycogen, glucagon, leupeptin, PMSF, polylysine (average M_r of 25,000), protamine, and TLCK were all from Sigma Chemical Co. (St. Louis MO). TLCK-treated trypsin was from Worthington. Coomassie Brillant Blue R-250 and BrCN-activated aminohexyl-Sepharose 4B were from Bio-Rad Labs. [γ^{32}P]ATP (5,800 Ci/mmol) was prepared as described previously (5).

Buffered solutions

Buffer A was 500 mM sucrose, 20 mM Tris-HCl (pH 7.2), 2 mM EDTA, 2 mM EGTA, 1 mM benzamidine, 0.5 mM TLCK, and 1 μg/ml leupeptin. Buffer B was 200 mM sucrose, 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 1 mM EGTA, and 1 mM benzamidine. Buffer B-T was buffer B plus 0.1% (v/v) Triton X-100. All these buffers contained, in addition, 1 mM PMSF and 0.5 mM DTT. Buffer C contained 40 mM Bistris (pH 6.5), 0.5 mg/ml bovine serum albumin, and 1 mM DTT.

Preparation of hepatic microsomes and hepatic glycogen-protein particles

Female Sprague-Dawley rats weighing 200–250 g were either fed ad libitum or fasted for 24 h. The latter animals were given 0.35 mg of glucagon intraperitoneally 30 min before death to achieve maximal glycogen depletion. The animals were killed by decapitation. The livers were rinsed and homogenized 1:3 (w/v) in ice-cold buffer A in a Potter-Elvejhem homogenizer. The homogenates were centrifuged for 15 min at 10,000 *g* at 4°C, and the postmitochondrial supernatant (PMS) was removed. The PMS was immediately centrifuged at 100,000 *g* for 90 min. at 4°C, leaving a supernatant (cytosol) and a pellet. The pellet was gently vortexed in the tube and the microsomal fractions were separated from the glycogen pellet. The microsomes and the glycogen pellet were resuspended separately in buffer B, both were recentrifuged at 100,000 *g* for 90 min at 4°C, and the supernatants were discarded. Washed microsomes were resuspended in buffer B to a final concentration of 25 mg/ml protein; the washed glycogen pellet was resuspended in a minimum volume of the same buffer.

Sucrose density gradient centrifugation

Aliquots of the PMS fraction obtained from fed rats were subjected to a linear sucrose-density gradient centrifugation essentially as described previously (15). In brief, aliquots (3 ml) of PMS fraction were carefully layered on top of a linear sucrose density gradient (15–60% w/v, 31 ml), containing Tris-HCl 5 mM (pH 7.4), 10 mM EDTA. After centrifugation at 25,000 rpm for 4 h at 4°C in a swing-out rotor (Beckman 28-SW), fractions of 0.85 ml were collected from the bottom of the centrifuge tube.

Solubilization of the HMG-CoA-Rd phosphatase activity from microsomes and glycogen protein pellet

Washed microsomal membranes and the washed glycogen-protein pellet were homogenized in a hand-operated glass homogenizer in the ratio 1:1 (v/w) with respect to the weight of original liver either with buffer B, buffer B-T, or buffer B plus KCl to a final concentration of 0.5 M. The suspensions were incubated for 1 h on ice, and centrifuged at 100,000 *g* for 90 min and the pellets were resuspended in the same homogenizing buffer. Alternatively, washed microsomal membranes and washed glycogen-protein pellets resuspended in buffer B were treated with α-amylase to a final concentration of 0.1 mg/ml and dialyzed at 4°C for 4 h against buffer B containing 1 μg/ml leupeptin. The suspensions were centrifuged at 100,000 *g* for 90 min to obtain the solubilized phosphatase. Control incubations were performed in which α-amylase was omitted.

Binding of solubilized microsomal HMG-CoA-Rd phosphatase to added purified glycogen

Liver glycogen was added to aliquots of detergent-solubilized microsomal extracts obtained with buffer B-T, from fed or fasted rats treated with glucagon, to final concentrations of 0.1, 0.3, 0.6, 1.5, and 10 mg/ml. After incubation for 30 min at 4°C, the suspensions were centrifuged for 90 min at 100,000 *g*. The supernatants

were removed, pellets were resuspended in 0.1 ml of the same buffer, and both fractions were assayed for HMG-CoA-Rd phosphatase activity. Control incubations were performed in which glycogen was omitted.

Protein preparations

Homogeneous HMG-CoA reductase (M_r 52,000), homogeneous labeled [^{32}P]HMG-CoA reductase from rat liver, [^{32}P]phosphorylase a from rabbit skeletal muscle, the catalytic subunits of protein phosphatase 1 and 2A, inhibitor-2 from rabbit skeletal muscle (I-2), the HMG-CoA-Rd phosphatase inhibitor (RP-inhibitor) from rat liver, and glycogen synthase kinase-3 from rabbit muscle were purified as described previously (14).

Assay methods

HMG-CoA reductase activity was assayed as described previously (16). One unit of HMG-CoA reductase is defined as the amount of enzyme catalyzing the conversion of 1 nmol of HMG-CoA to mevalonate in 1 min at 37°C.

Prior to assay of protein phosphatase activity, the initial extracts were filtered through a Sephadex G-25 column (1.5 × 5 cm) equilibrated in buffer B. The liver fractions were assayed at maximum dilutions.

HMG-CoA reductase phosphatase activity was determined by the increase in HMG-CoA reductase activity acting on the inactivated homogeneous enzyme, and the results were compared with a control without phosphatase. The assay was carried out as follows: 0.03 ml of protein phosphatase was incubated with 0.02 ml of homogenous HMG-CoA reductase (0.22 μg) in buffer C. After 15 min at 37°C, the HMG-CoA reductase phosphatase activity was determined as previously described by Hegardt, Gil, and Calvet (17). One unit of HMG-CoA-Rd phosphatase is defined as the amount of enzyme that increases the activity of HMG-CoA reductase by 1 milliunit in 1 min at 37°C.

Phosphorylase phosphatase activity was determined by measuring the release of ^{32}P from ^{32}P-labeled phosphorylase a (1 mg/ml) at 30°C as described by Khandelwal, Vandenheede, and Krebs (18). One unit of phosphorylase phosphatase activity is defined as the amount of enzyme that catalyzes the release of 1 nmol [^{32}P]phosphate per min.

When the effect of inhibitor-2 from rabbit skeletal muscle was investigated to classify HMG-CoA-Rd phosphatases as type 1 or type 2, the initial fractions were preincubated with inhibitor-2 (0.15 μg/ml) for 10 min at 37°C. Inhibitor-2 was assayed for its ability to inhibit protein phosphatase 1 using [^{32}P]phosphorylase a as substrate according to the method of Foulkes and Cohen (19). One unit of inhibitor-2 decreases the activity of 15 milliunits of protein phosphatase 1 by 50% in the standard phosphorylase phosphatase assay.

To reactivate type 2A phosphatase activity, the assay was performed in the presence of 25 μg/ml of protamine as described by Pelech and Cohen (20). The specific assay for type 2C protein phosphatase activity was carried out as described by Ingebritsen, Foulkes, and Cohen (21), using HMG-CoA reductase as substrate. The specific assay for type 2B protein phosphatase activity was carried out as described previously (22) using *p*-nitrophenyl phosphate as the substrate.

Glucose-6-phosphatase (23) and the contribution of nonspecific phosphatase (24) were assayed as previously described; the difference between total and nonspecific phosphohydrolase activities was taken as the true glucose-6-phosphatase activity. Lactate dehydrogenase activity was assayed as previously described (25). Protein concentration was determined by the method of Bradford (26) and bovine serum albumin was used as the standard. Glycogen was determined as described previously (27). RNA was determined as described by Reinhart et al. (1). SDS-polyacrylamide slab gels (7.5%) were made according to Laemmli (28) and stained with 0.25% Coomassie Brillant Blue.

Purification of HMG-CoA-Rd phosphatase activity from hepatic microsomes

Washed microsomes obtained from 140 g of liver from fed rats were solubilized with buffer B-T and the soluble fraction was chromatographed in a DEAE-cellulose column equilibrates with buffer B (5). The HMG-CoA-Rd phosphatase activity excluded from this column, called PP-R, was chromatographed in a column of phosphocellulose, and the active fractions were then chromatographed in an aminohexyl-Sepharose 4B column as previously described (5). The pool of active fractions was concentrated and desalted by ultrafiltration using an Amicon cell with PM-10 filter and buffer B. The concentrated fractions were applied to a polylysine-Sepharose 4B (2 × 6 cm) column equilibrated with buffer B, and the phosphatase activity was eluted using 200 ml linear gradient of 0–0.5 M KCl in buffer B.

Other assays

When indicated, the fractions excluded from the DEAE-cellulose column were alternatively treated with trypsin, ethanol, acetone, or KCl to dissociate the catalytic subunit of other proteins from the holoenzyme. Digestions with trypsin were carried out in a trypsin/protein ratio (1:90 w/w) for 10 min at 30°C, in the presence or absence of 5 mM Mn^{2+}, and this process was terminated by the addition of trypsin-inhibitor in an inhibitor/trypsin ratio (10:1 w/v). Ethanol treatment of PP-R (29) and acetone precipitation (30) were carried out as described earlier. Treatment with KCl was performed by incubating the fractions excluded from DEAE-cellulose column for 1 h at 4°C with solid KCl to a final concentration of 0.5 M.

RESULTS

Subcellular distribution of HMG-CoA-Rd phosphatase activity in livers from fed or fasted rats.

Most of the HMG-CoA-Rd phosphatase activity of the rat liver postmitochondrial supernatant was present in the cytosolic fraction in fed animals (**Table 1A**) and much less was found in microsomal membranes and glycogen. When animals were fasted for 24 h and treated with glucagon to deplete the glycogen content, there was no change in total HMG-CoA-Rd phosphatase activity, but there was a movement of the glycogen-bound phosphatase activity into the cytosol together with a decrease (3- to 4-fold) in that bound to microsomes (Table 1B). The microsomal phosphatase activity under these conditions appeared to be about 2% of the initial activity but it was not due to glycogen contamination. The use of the cytosolic marker lactate dehydrogenase ensured that this small amount of microsomal phosphatase was not produced by cytosolic contamination. In addition, the microsomal preparation was not contaminated with phosphatases from the plasma membranes, as 5' nucleotidase activity was not observed (data not shown).

The subcellular location of HMG-CoA-Rd phosphatase was also examined by subjecting the PMS fraction to linear sucrose density gradient centrifugation. Under these conditions the glycogen pellet remained at the bottom of the centrifuge tube. As shown in **Fig. 1A** the HMG-CoA-Rd phosphatase activity exhibited a dual distribution. Most of the activity was associated with the cytosolic marker protein lactate dehydrogenase and a second minor peak coincided with glucose-6-phosphatase and HMG-CoA reductase activities, both marker proteins of the endoplasmic reticulum (Fig. 1B). The percentage of HMG-CoA-Rd phosphatase activity bound to microsomal membranes appeared to be the same whether the subcellular fractionation or sucrose density centrifugation procedure was used.

Solubilization of HMG-CoA-Rd phosphatase activity from microsomes and the glycogen pellet

To study the HMG-CoA-Rd phosphatase activity, washed microsomal fractions were extracted with 0.1% Triton X-100. More than 75% of total activity was extracted in the first step (**Fig. 2A**); this increased to 95% after three extractions. When the extraction was carried out with 0.5 M KCl, similar yields were achieved. HMG-CoA-Rd phosphatase activity was not affected by either of these treatments. Identical solubilization procedures performed on the glycogen pellet produced a reduced solubilization of phosphatase activity (30% on Triton X-100 treatment and 28% with KCl).

When the glycogen pellet was solubilized with α-amylase, almost 100% of phosphatase activity was solubilized, in sharp contrast with the low solubilization of microsomal phosphatases under this treatment compared with control without α-amylase (Fig. 2B). When microsomes were resuspended in buffer B (control experiment), about 40% of the HMG-CoA-Rd phosphatase activity was released into the supernatant. This is in close agreement with data presented by different authors for systems other than HMG-CoA-Rd phosphatase activity (13, 31). The ease of solubilization of microsomal protein phosphatase by treatment with buffer B only suggests that this phosphatase is not an integral membrane protein. In contrast, little HMG-CoA-Rd phosphatase activity was released from the glycogen fraction incubated with buffer B.

TABLE 1. Distribution of HMG-CoA-Rd phosphatase activity in the postmitochondrial supernatant (PMS)

Fraction	Lactate Dehydrogenase	Glucose-6-Phosphatase	Phosphorylase Phosphatase	HMG-CoA-Rd Phosphatase
A) Fed				
PMS[a]	263 ± 30	3.24 ± 1.2	50 ± 8	1700 ± 250
	%[b]	%	%	%
PMS	100	100	100	100
Cytosol	90	2.5	82	55
Microsomal fraction	0.5	80	12	7
Glycogen fraction	0.4	4	7	6
B) Fasted, glucagon-treated				
PMS[a]	267 ± 25	3.54 ± 1.4	47 ± 6	1900 ± 180
	%[b]	%	%	%
PMS	100	100	100	100
Cytosol	96	2	89	62
Microsomal fraction	0.4	72	4	2

The distribution of the HMG-CoA-Rd phosphatase, glycogen phosphorylase phosphatase, glucose-6-phosphatase, and lactate dehydrogenase activities in the three cell fractions that make up the postmitochondrial supernatant is shown. The PMS were prepared from livers of either fed (A) or fasted, glucagon-treated (B) animals. In fed and glycogen-depleted animals, the glycogen concentrations were 41 ± 10 mg/g and 0.2 ± 0.1 mg/g liver, respectively.

[a]Units/g of liver ± SEM ($n = 3$).

[b]Data are percentage of the PMS values recovered in other fractions.

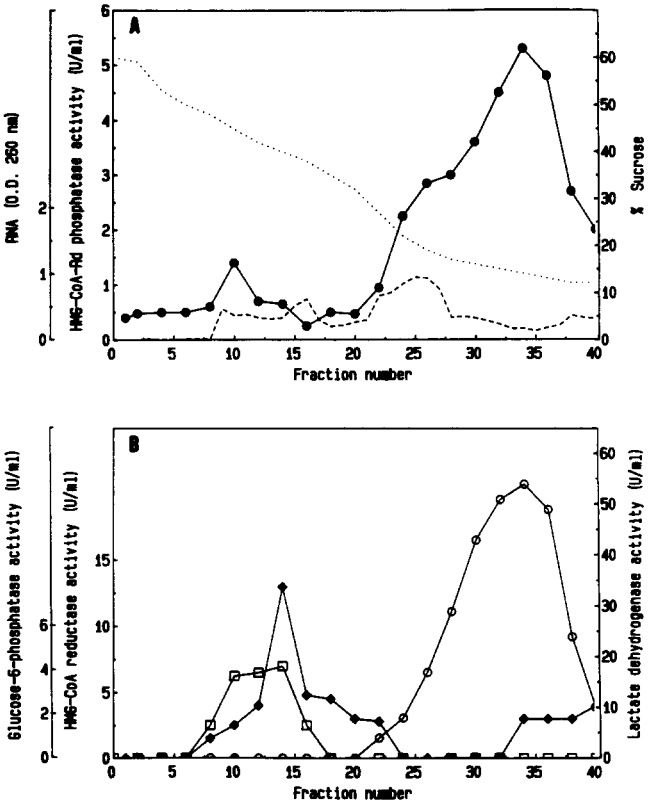

Fig. 1. Distribution of HMG-CoA-Rd phosphatase activity in a linear sucrose density gradient. The postmitochondrial supernatant fraction obtained from fed animals was subjected to a linear sucrose density gradient centrifugation as described in Materials and Methods. Fractions of 0.85 ml were taken and aliquots of each fraction were assayed for HMG-CoA-Rd phosphatase (●), HMG-CoA reductase (♦), glucose-6-phosphatase (□), and lactate dehydrogenase (○) activities. RNA (-----) was determined as described in Materials and Methods. The density of sucrose is expressed in percentage (w/v) (....).

These experiments show that phosphatase activity bound to microsomal membranes exhibits solubilization properties different from those of the phosphatase activity bound to glycogen.

The assay of binding to purified glycogen of the HMG-CoA-Rd phosphatase activity solubilized from microsomal membranes indicates that the activity is fully recovered in the supernatant fraction, in assays both with fed rats and with fasted rats treated with glucagon (data not shown), from which we concluded that there is no affinity of the microsomal solubilized phosphatases with glycogen at variance with what happens with glycogen synthase phosphatase (11).

Several protein phosphatases are present in microsomal membranes

In order to characterize the HMG-CoA-Rd phosphatase present in microsomal membranes, the Triton X-100-solubilized fractions were chromatographed on a DEAE-cellulose column and then assayed by either a standard HMG-CoA-Rd phosphatase activity or by specific assays for type 1, 2A, 2B, and 2C protein phosphatases. Several peaks with HMG-CoA-Rd phosphatase activity were obtained when the assay was carried out using a standard procedure (**Fig. 3A**). The first peak corresponding to the phosphatase activity not retained by the column was not activated by protamine and was slightly inhibited by Mg^{2+} (Fig. 3B). As activation by protamine is a characteristic of type 2A protein phosphatases, and as activation by Mg^{2+} is characteristic of type 2C phosphatase, it may be concluded that the excluded HMG-CoA-Rd phosphatase activity is a non-2A, non-2C protein phosphatase.

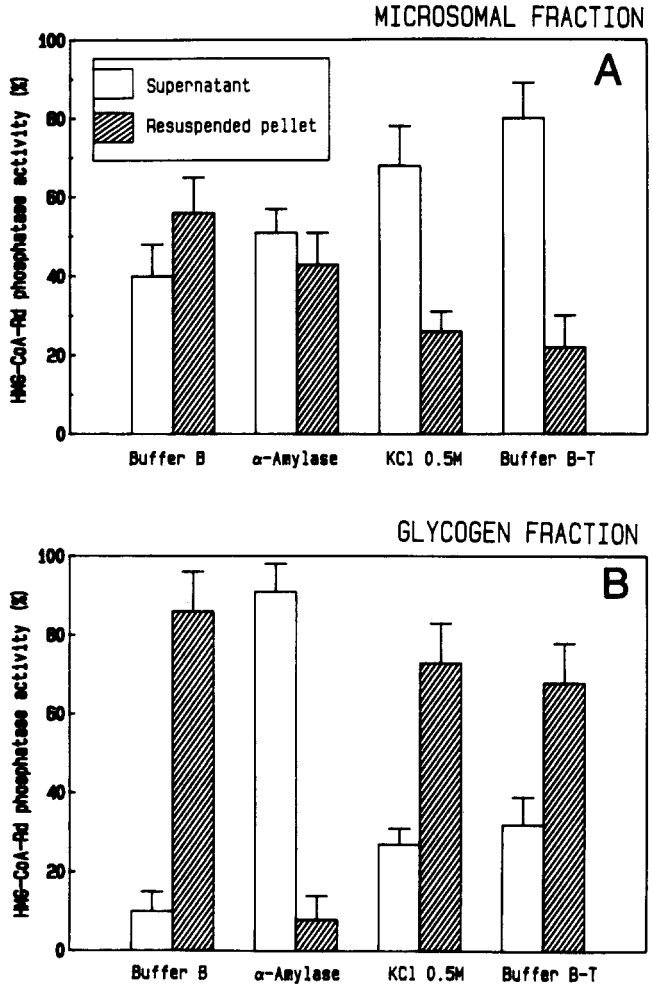

Fig. 2. Solubilization of HMG-CoA-Rd phosphatase activity from microsomal membranes and glycogen protein complex. Microsomal and glycogen pellet fractions obtained from fed rats were incubated 1 h on ice with buffer B, buffer B-T, and buffer B with 0.5 M KCl. Other aliquots were incubated with α-amylase and then dialyzed against buffer B at 4°C for 4 h (see Materials and Methods). Treated samples were centrifuged at 100,000 *g* for 90 min, and the HMG-CoA-Rd phosphatase activity was assayed in the supernatant and in the resuspended pellet. Results are expressed as percentage of activity with respect to the initial activity.

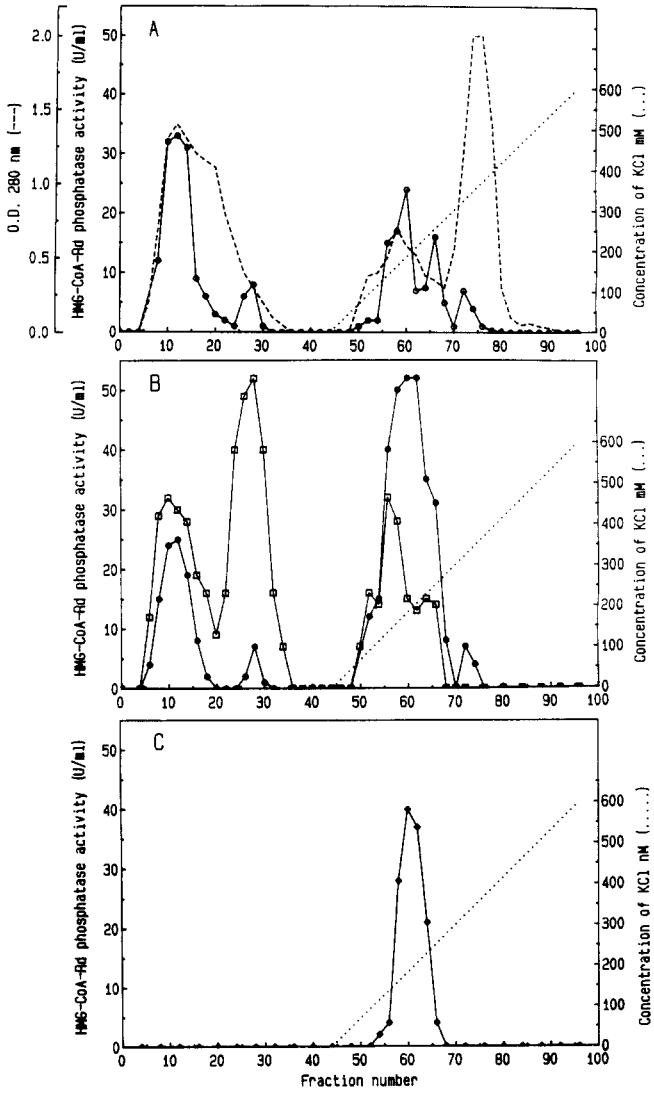

Fig. 3. DEAE-cellulose chromatography of solubles from rat liver microsomes treated with buffer B-T. Liver microsomes obtained from five fed rats were solubilized with buffer B-T and then chromatographed in a DEAE-cellulose column (2.5 × 6 cm), equilibrated with buffer B. after washing, the gradient 0–0.6 M KCl (200 + 200 ml) was set and 8-ml fractions were collected. A. The standard HMG-CoA-Rd phosphatase assay was performed (○). B. HMG-CoA-Rd phosphatase activity was assayed in the presence of 30 μg/ml of protamine (□) or in the presence of 5 mM $MgCl_2$ (●). C. Aliquots of the samples were incubated with 50 mM NaF, 10 mM EDTA (pH 7.0) for 60 min at 30°C; then, samples were dialyzed against buffer B to remove NaF and EDTA, and thereafter the HMG-CoA-Rd phosphatase assay was carried out (♦).

The phosphatase activity eluted from the column after the buffer washing was 4- to 5-fold reactivated by protamine treatment and, moreover, it was not affected by the presence of 5 mM Mg^{2+} (Fig. 3B). These data suggest that it was a type 2A phosphatase. The third peak corresponding to a 0.15 M KCl concentration showed a slight reactivation pattern under protamine treatment, suggesting that it was a different kind of type 2A phosphatase. Lastly, the peak of phosphatase activity corresponding to a gradient concentration of 0.25 M KCl was activated 2- to 3-fold in the presence of 5 mM Mg^{2+} (Fig. 3B), without any appreciable reactivation pattern under protamine treatment. The specific assay for type 2C phosphatase activity that involves the permanent inactivation of any other protein phosphatase activity, showed enzymatic activity associated only to the fourth peak (0.25 M KCl) (Fig. 3C). When all fractions were assayed in the presence of I-2 (0.15 μg/ml), their phosphatase activities were not changed when either HMG-CoA reductase or glycogen phosphorylase was the substrate (data not shown).

Calcineurine (protein phosphatase type 2B obtained from Sigma) was able to reactivate the HMG-CoA reductase activity and this reactivation increased in the presence of Ca^{2+}/calmodulin (data not shown). However, no change in activity was observed when all DEAE-cellulose fractions were incubated in the presence of 0.2 mM Ca^{2+} and 10 μM calmodulin, when either HMG-CoA reductase or *p*-nitrophenyl-phosphate was the substrate. Thus, protein phosphatase activity that is excluded first from the DEAE-cellulose column, which we referred to as non-2A, non-2C, is also non-2B protein phosphatase. We shall refer to this activity as PP-R.

Partial purification of the HMG-CoA-Rd phosphatase excluded from DEAE-cellulose column (PP-R)

The microsomal protein phosphatase (PP-R) excluded from the DEAE-cellulose column was purified further as described by Sitges, Gil, and Hegardt (5). Briefly, the active phosphatase fractions were successively chromatographed on phosphocellulose and aminohexyl-Sepharose 4B columns. Material from the aminohexyl-Sepharose 4B column (540 units) was chromatographed on a polylysine-Sepharose 4B column as described in Materials and Methods. A small part of the activity was excluded and most activity was eluted with 0.1 M KCl. The active fractions were concentrated by ultrafiltration (125 units) and chromatographed in a Bio-Gel A-0.5 m column (1.5 × 80 cm) equilibrated with buffer B plus 0.1 M KCl. A single peak of HMG-CoA-Rd phosphatase activity was observed corresponding to a protein with an apparent molecular mass of 85 kDa. At this level of purification, the SDS-PAGE of active fractions after Coomassie staining, showed two major bands corresponding to molecular masses of 54 and 48 kDa.

Dephosphorylation and reactivation of ^{32}P-labeled HMG-CoA reductase

In order to ascertain whether the reactivation of HMG-CoA reductase caused by PP-R was indeed bound to its dephosphorylating action, a dephosphorylation experiment was carried out using homogenous [^{32}P]HMG-CoA reductase as the substrate. Results shown in **Fig. 4A** indicate that when the time of incubation increases, more

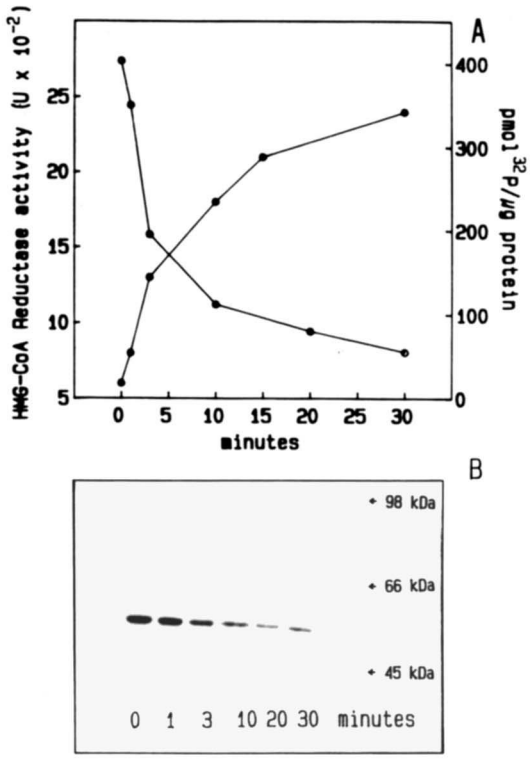

Fig. 4. Dephosphorylation of [^{32}P]HMG-CoA reductase by PP-R. A. Aliquots (0.18 ml, 6.24 µg) of homogeneous [^{32}P]HMG-CoA reductase (1.20 µCi/mg) were incubated with PP-R obtained after purification in aminohexyl-Sepharose 4B in a final volume of 0.7 ml; aliquots (0.005 ml) of incubation mixture were taken at the indicated time and diluted with Tris-HCl 40 mM (pH 7), 1 mM DTT, 5 mM PPi, 50 mM KF, and bovine albumin 0.5 mg/ml. The percentage of HMG-CoA reductase activity is represented on the ordinate (●). Other aliquots (0.035 ml) from the reaction mixture were measured for the protein-bound ^{32}P (5). The picomols of ^{32}P bound to HMG-CoA reductase are shown to the right (○). B. Aliquots (0.045 ml) from the incubation mixture described in A were electrophoresed in SDS-PAGE (7.5%) and analyzed by autoradiography.

^{32}P is released from the HMG-CoA reductase and that this effect is concomitant with the increase of HMG-CoA reductase activity. Aliquots from the previous experiment were electrophoresed on SDS-PAGE and analyzed by autoradiography. The results show a correlative decrease of the radioactive mark corresponding to [^{32}P]HMG-CoA reductase (Fig. 4B).

Characterization of HMG-CoA-Rd phosphatase (PP-R)

The preparation excluded from DEAE-cellulose was assayed for inhibitor-2 activity by boiling 0.2 ml for 10 min, and centrifuging for 2 min at 15,000 *g*; 0.015 ml of supernatant was tested for its inhibitory activity by incubation with 0.015 ml of catalytic subunit type 1 from rabbit skeletal muscle (15 milliunits) and 0.02 ml of glycogen phosphorylase (1 mg/ml) as substrate, at 30°C for 15 min. Results showed that no inhibitory activity corresponding to I-2 was detected. In addition, incubation for 30 min at 30°C of 0.015 ml of the preparation excluded from DEAE-cellulose with 2 mM magnesium acetate, 0.2 mM ATP, and 5 µg of glycogen synthase kinase-3 in a total volume of 0.03 ml failed to reactivate the HMG-CoA-Rd phosphatase activity. It is concluded from the two experiments that the purified phosphatase preparation did not contain inhibitor-2 protein.

Partially purified PP-R obtained after the chromatography in polylysine-Sepharose 4B was less inhibited than the rabbit skeletal muscle catalytic subunit type 1 phosphatase at the same concentration of I-2, when HMG-CoA reductase was the substrate (**Fig. 5A**). The calculated IC_{50} was 30 nM for PP-R, and 3 nM for the type 1 phosphatase (these calculations were performed

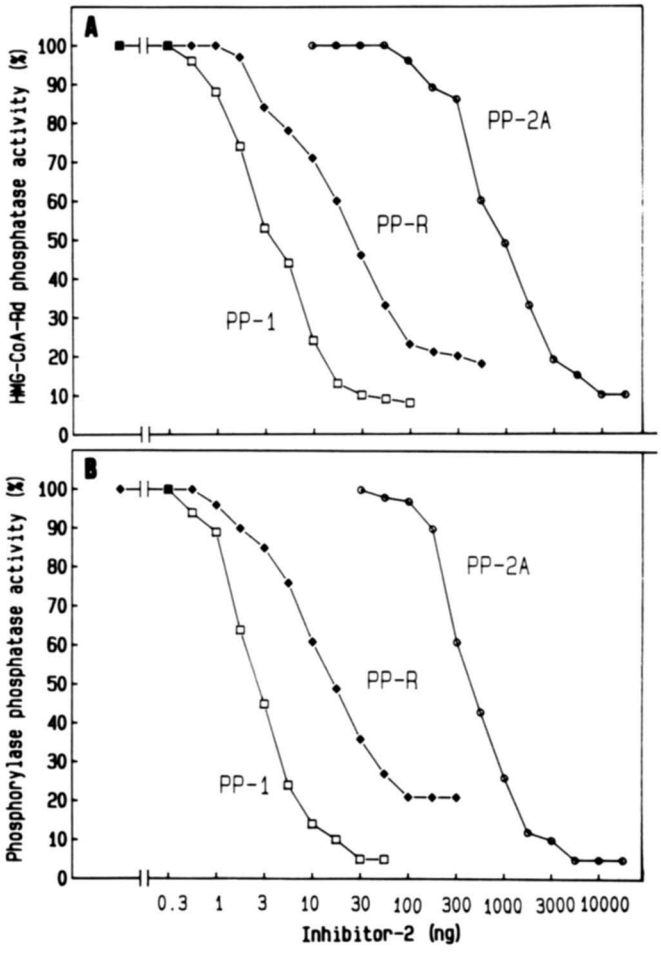

Fig. 5. Effect of the inhibitor-2 on the PP-R activity. A. Aliquots from concentrated preparations from chromatography on polylysine Sepharose-4B (0.015 ml, 4 units) (♦) were incubated at 37°C for 15 min with different amounts of inhibitor-2, in a final volume of 0.030 ml, and the phosphatase activity was measured using HMG-CoA reductase as substrate. The same assay was carried out with type 1 (□) and type 2A (○) catalytic protein phosphatases from rabbit skeletal muscle. B. Other aliquots from the same preparations were assayed using glycogen phosphorylase as substrate. The percentage of protein phosphatase activity is represented on the ordinate. Symbols are the same as in A.

using a molecular mass of 22,835 daltons for I-2, as deduced from sequence analysis) (32). Moreover, saturating concentrations of I-2 did not produce full inhibition of PP-R, about 20% of the initial activity remaining. These results were obtained not only with HMG-CoA reductase but also with phosphorylase as the substrate (Fig. 5B).

As evidence that purified protein phosphatase is not contaminated by protein phosphatase 2A, samples from polylysine-Sepharose 4B chromatography (4 units) were incubated with 25 μg/ml protamine for 30 min. Not only was the preparation not reactivated, but its activity fell to 70% of its initial level, showing the absence of type 2A protein phosphatase in the preparation (20). When the inhibitory assay was carried out in the presence of HMG-CoA-Rd phosphatase inhibitor (RP-inhibitor) a slight inhibition was produced which was quite different from that with protein phosphatase 2A (**Fig. 6**).

Both series of results taken together suggest that PP-R does not contain PP-2A and is a type of protein phosphatase 1 with lower sensitivity to inhibitor-2 than cytosolic type 1 protein phosphatase from rabbit skeletal muscle.

Subunit composition PP-R

In order to further characterize the PP-R microsomal enzyme excluded from DEAE-cellulose, several gel filtration experiments were carried out on Bio-Gel A-0.5m. PP-R obtained after DEAE-cellulose chromatography was treated either with trypsin (in presence or absence of Mn^{2+}), acetone, or ethanol. All these treatments decreased HMG-CoA-Rd phosphatase activity, 10–15% of the initial activity remaining in all three cases. When the residual activity obtained after trypsinization of PP-R was chromatographed on the Bio-Gel A-0.5m column, the elution position of the phosphatase activity indicated a maximum peak corresponding to an apparent molecular mass of 37 kDa (**Fig. 7A**). The same chromatography peak was obtained with material resulting from acetone precipitation or ethanol treatment.

When the PP-R obtained after DEAE-cellulose column was treated with solid KCl to a final concentration of 0.5 M KCl, no change in the enzyme activity was ob-

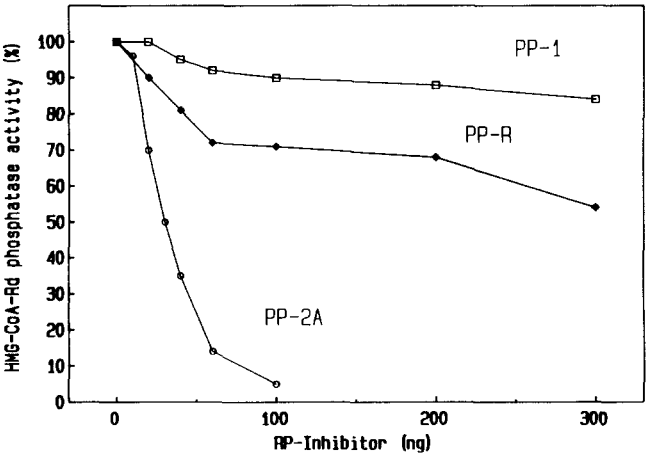

Fig. 6. Effect of RP-inhibitor on the PP-R activity. Aliquots with HMG-CoA-Rd phosphatase activity (0.015 ml, 4 units) (♦) from concentrated preparations from chromatography on polylysine-Sepharose 4B were incubated at 37°C for 15 min with separate amounts of RP-inhibitor in buffer C in a final volume of 0.03 ml. Similar assays were carried out with preparations of the catalytic subunit type 1 (□) and catalytic type 2A protein phosphatases (○) from rabbit skeletal muscle. The percentage of HMG-CoA-Rd phosphatase activity is represented on the ordinate.

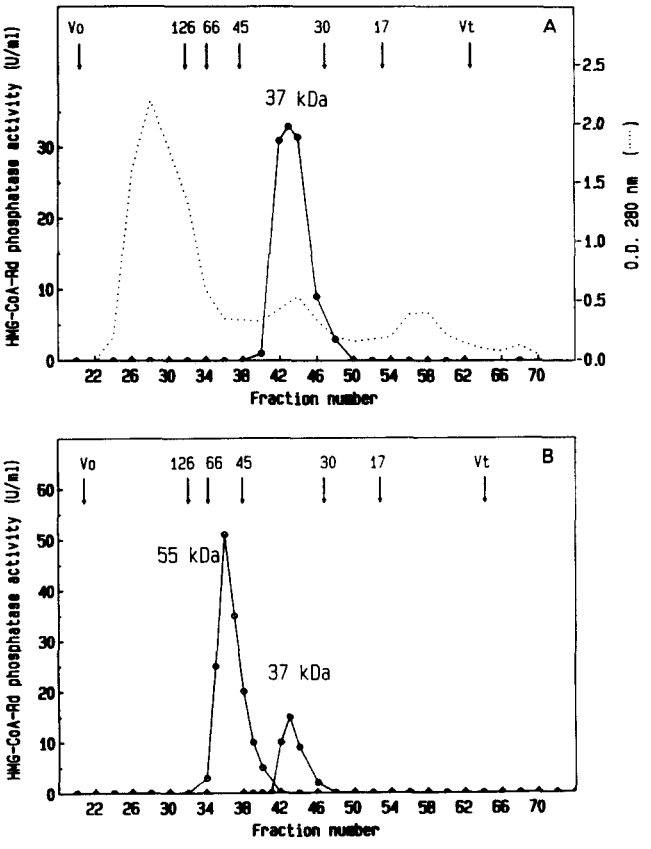

Fig. 7. Gel filtration of PP-R treated under different procedures. The soluble fractions obtained by Triton X-100 treatment of microsomes from 100 g of rat liver were applied to a DEAE-cellulose column. Excluded fractions with phosphatase activity were pooled and treated differently; A. An aliquot was treated with trypsin (as described in Materials and Methods), concentrated to 10 ml, and applied (880 units) to a Bio-Gel A-0.5m column (2.5 × 55 cm) equilibrated in buffer B + 0.1 M KCl; HMG-CoA-Rd phosphatase activity (●) was measured. Absorbance at 280 nm (....) is represented on the ordinate. The flow rate was 10 ml/h, and fractions of 4 ml were collected. Molecular weight standards were: ferritin 440,000 (Vo); alcohol dehydrogenase 126,000, bovine serum albumin 66,000, ovalbumin 45,000, carbonic anhydrase 30,000, myoglobin 17,000 and potassium dichromate (Vt). B: Another aliquot was treated with 0.5 M KCl and applied to the same column and chromatographed in the same conditions as described above. HMG-CoA-Rd phosphatase activity (○) was measured. Another KCl-treated aliquot was incubated with trypsin (as described in Materials and Methods) and applied to the same column. HMG-CoA-Rd phosphatase activity (●) was measured. Relative molecular mass standards were the same as described in A.

served. An aliquot was chromatographed on a Bio-Gel A-0.5 m column and a peak with HMG-CoA-Rd phosphatase activity corresponding to a molecular mass of 55 kDa was observed. Another aliquot was treated with trypsin and thereafter chromatographed on a Bio-Gel A-0.5m column. A phosphatase activity peak bound to a protein of 37 kDa was observed (Fig. 7B).

Lastly, the solubilized 0.5 M KCl fractions from washed microsomes obtained after centrifugation at 100,000 *g* for 60 min were dialyzed in buffer B for 12 h, chromatographed on a DEAE-cellulose column, and assayed for HMG-CoA-Rd phosphatase activity. Most of the activity was excluded from the column and only a small part was retained with a distribution pattern similar to that shown in Fig. 3A, in which microsomes were solubilized with Triton X-100. When the activity PP-R excluded from DEAE-cellulose was chromatographed on Bio-Gel A-0.5m, a single peak of HMG-CoA-Rd phosphatase activity corresponding to a molecular mass of 55 kDa was observed. Treatment of these fractions with trypsin followed by Bio-Gel A-0.5m chromatography yielded a phosphatase activity bound to a protein of 37 kDa. Profiles of 55 kDa and 37 kDa proteins on the Bio-Gel A-0.5m column were exactly like those of the Fig. 7B and they are not shown.

All these results taken together suggest that the protein phosphatase activity may be associated with different holoenzymatic forms. A protein of 85 kDa may be dissociated into a catalytically active 55 kDa form and a nonenzymatic form that probably facilitates the binding of the phosphatase in the microsomes. In addition, a 37 kDa enzymatically active phosphatase may be derived from higher forms: the 85 kDa and the 55 kDa phosphatases.

DISCUSSION

This paper reports the occurrence of HMG-CoA-Rd phosphatase activity bound to the endoplasmic reticulum. Arguments to support this idea are several: *a*) depletion of glycogen in liver does not fully eliminate the HMG-CoA-Rd phosphatase activity present in microsomes, a constant 2% of the initial activity remains; *b*) the profile of HMG-CoA-Rd phosphatase activity obtained after centrifugation of the postmitochondrial supernatant in a continuous gradient of sucrose presents a dual distribution between endoplasmic reticulum and cytosol; *c*) different patterns of phosphatase activity are obtained upon solubilization of microsomes and glycogen pellet by different tensoactive buffers; and *d*) attempts to bind HMG-CoA-Rd phosphatase to glycogen at various concentrations, under the conditions described by Mvumbi and Stalmans (11) that there is no affinity of this phosphatase for glycogen.

We have previously reported the occurrence in microsomes of several protein phosphatase activities (5); later Diven et al. (33) observed a similar chromatography pattern on DEAE-cellulose of protein phosphatases of microsomal origin. However, no specific assays were carried out to exclude the possibility that these phosphatase activities could be due to glycogen contamination. Various authors have studied the distribution of glycogen synthase phosphatase in glycogen and microsomes (11–13, 34). While it remains doubtful whether glycogen synthase phosphatase activity is present in microsomes, several groups agree that microsomes contain phosphorylase phosphatase activity (34, 35). The occurrence of phosphorylase phosphatase as an enzyme present in microsomes supports the notion that HMG-CoA-Rd phosphatase may actually be present as a microsomal activity. We have presented evidence of the occurrence of several protein phosphatases in microsomal extracts, namely a type 2A, a type 2C, and a protein that behaves as non-2A, non-2B, and non-2C.

The occurrence of a type 1 protein phosphatase in microsomes has been reported previously (35). This fact and the impossibility of classifying the activity excluded by the DEAE-cellulose column as a type 2 protein phosphatase led us to study this activity more carefully. In this article we report the partial purification of PP-R. The partially purified protein is totally active, as incubation with glycogen synthase kinase-3, protamine, or with Mg^{2+} failed to increase its spontaneous activity. The PP-R was inhibited by small concentrations of I-2 and was not affected by the RP-inhibitor. On the basis of these properties, this microsomal protein may be classified as a type 1 protein phosphatase with lower sensitivity to I-2 than other type 1 protein phosphatases. This lower sensitivity to I-2 of the native forms of phosphorylase phosphatase of microsomal origin has been reported previously (31, 35), but none of those studies presented a purification procedure for either the holoenzyme or the catalytic subunit.

Results of solubilization by detergent and KCl and those of proteolysis allow us to propose a model for PP-R bound to microsomes, **Fig. 8**. This model would consist of: *a*) a type 1 catalytic subunit (C) similar to that described for other subcellular fractions; *b*) another regulatory subunit (R); and *c*) an endoplasmic reticulum linking subunit (E_r). Association of catalytic and regulatory proteins (CR) would have a molecular mass of 55 kDa in gel filtration. This complex (CR) is easily removed from microsomes by treatment with KCl. These results for HMG-CoA-Rd phosphatase are in agreement with Alemany et al. (31): a molecular mass of 50–60 kDa for the protein phosphatase activity from the same microsomal origin was measured when glycogen synthase and glycogen phosphorylase were the substrates. The whole model (CRE_r) of 85 kDa could only be solubilized by treatment

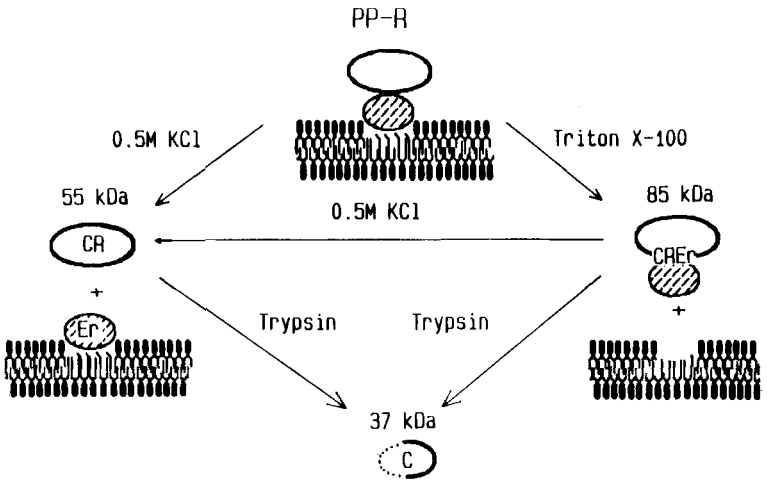

Fig. 8. Multi-subunit proposed model of protein phosphatase (PP-R) bound to microsomal membranes. The protein phosphatase (PP-R) is bound to the endoplasmic reticulum membrane through the E_r subunit. The catalytic C subunit is the one that contains enzymatic activity. The R subunit provides the maximum catalytic activity for the C subunit. The holoenzyme CRE_r is easily solubilized with Triton X-100. The KCl extraction dissociates the E_r subunit. All forms produce the 37 kDa fragment by proteolytic treatment.

with detergent and would be constituted by the three subunits. This complex would in turn be split by the 0.5 M KCl treatment, producing the CR complex with catalytic activity and the E_r subunit, without activity.

Treatment with proteolytic systems (ethanol, trypsin, acetone) inactivated most of the HMG-CoA-Rd phosphatase activity (85–90%). These results indicate that, in addition to a catalytic subunit, the microsomal protein phosphatase requires a (trypsin-sensitive) "specifying subunit" (R) in order to recognize HMG-CoA reductase as the substrate. An analogous system has been proposed for the glycogen-bound glycogen synthase phosphatase activity by Bollen et al. (36). The modulator protein (inhibitor-2) does not apparently form part of the type 1 microsomal protein. This is deduced from the negative results of phosphorylation in the presence of glycogen synthase kinase-3 and also from the absence of inhibitory activities after heating the preparation to 100°C.

Cohen and co-workers (37) reported that the amino acid sequence from rabbit liver protein phosphatase catalytic subunit type 1 was identical to that from rabbit skeletal muscle. In addition, these authors also showed a high level of homology (93% identical amino acid residues) between the mammalian and the insect type 1 protein phosphatase (38), suggesting that distinctive substrate specificity and regulatory properties are not conferred by the catalytic subunits themselves, but rather by regulatory subunits that are combined with the catalytic subunits in vivo. This model could also be applied to the interaction between the rat liver type 1 microsomal protein phosphatases, PP-R, and the HMG-CoA reductase. This report proposes that a specifying subunit bound to the catalytic subunit increases the phosphatase activity. Further, the presence of another E_r protein appears to be responsible for anchoring the CR complex to the endoplasmic reticulum as a consequence of its role in cholesterol biosynthesis regulation.

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