Purification and characterization of a protein inhibitor from rat liver that inhibits type 1 protein phosphatase when 3-hydroxy-3-methylglutaryl CoA reductase is the substrate

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Abstract A protein inhibitor of HMG-CoA reductase phosphatase activity from rat liver was purified to homogeneity. The protein was purified 4,000-fold with an overall yield of 4%. The purified protein had a molecular mass of 31 kDa. This spontaneously active protein is thermostable and acid-resistant. The protein inhibitor is phosphorylated by glycogen synthase kinase-3 and cAMP-dependent protein kinase without change in its inhibitory activity. The inhibition caused by this inhibitor on phosphatases 1 and 2A is similar to that of inhibitor-2 from rabbit skeletal muscle using hydroxymethylglutaryl-CoA reductase as substrate. The regulation properties of this inhibitor towards phosphatase 1 together with another protein inhibitor of phosphatase 2A in cholesterol metabolism are discussed. —**Serra, D., G. Asins, and F. G. Hegardt.** Purification and characterization of a protein inhibitor from rat liver that inhibits type 1 protein phosphatase when 3-hydroxy-3-methylglutaryl CoA reductase is the substrate. *J. Lipid Res.* 1990. **31:** 919–926.

Supplementary key words HMG-CoA reductase phosphatases • protein phosphorylation • inhibitor-2

3-Hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase (E.C. 1.1.1.34) the main rate-limiting enzyme for synthesis of cholesterol and isoprenoids, has been shown to be regulated by covalent modification. Reductase kinase inactivates and phosphorylates HMG-CoA reductase both in vitro and in vivo, this process being activated by AMP (1, 2). Inactivated, homogeneous ^{32}P-labeled HMG-CoA reductase is reactivated in vitro by rat liver type 1 and type 2 protein phosphatases with a concomitant release of ^{32}P (3).

Type 1 protein phosphatases are inhibited at nanomolar concentration by the heat-stable proteins, termed inhibitor-1 and inhibitor-2 (4–6). In a different way, type 2 protein phosphatases are inhibited at much higher concentrations by inhibitor-2 (7). Inhibitor-1 described by Huang and Glinsman (4) requires phosphorylation by the cyclic AMP-dependent protein kinase for its activity. In contrast, inhibitor-2 is active without phosphorylation and usually copurifies with the phosphatase 1 as an inactive complex with a molecular mass of 70 kDa (8, 9).

Rabbit liver inhibitor-2 was purified to apparent homogeneity by Khandelwal and Zinman (10), and had an apparent molecular mass of 15 kDa by SDS-PAGE. Later, Chisholm and Cohen (11) reported that the partially purified inhibitor-2 from rabbit liver had the same molecular mass (30.5 kDa) as the inhibitor-2 from rabbit skeletal muscle.

There is no previous report on the purification to homogeneity of a rat liver protein inhibitor active on protein phosphatase 1. In this report, we describe a procedure for the purification of a protein inhibitor of phosphatase type 1 from rat liver using HMG-CoA reductase as the substrate. The purified protein was homogeneous as judged by polyacrylamide gel electrophoresis in the presence of SDS. The molecular mass of the rat liver inhibitor determined by SDS-polyacrylamide gel electrophoresis was found to be 31 kDa. This protein inhibitor showed kinetic properties towards protein phosphatases 1 and 2A similar to rabbit muscle inhibitor-2 using as substrates not only HMG-CoA reductase but also phosphorylase.

MATERIALS AND METHODS

Chemicals

Most of the reagents used in this study have been reported previously (12). cAMP-dependent protein kinase,

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; PMSF, phenylmethylsulfonyl fluoride; Bistris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)-1,3-propane-diol; TLCK, N-α-*p*-tosyl-L-lysine chloromethyl ketone; Brij 35, polyoxyethylene 23-lauryl ether; DTT, dithiothreitol; RP, HMG-CoA reductase phosphatase.

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benzamidine, leupeptin, PMSF, TLCK, and Brij 35 were from Sigma. Blue Sepharose CL 6B was from Pharmacia. Coomassie Brilliant Blue R-250, Agarose Low Gel Temperature, SDS, acrylamide, and bisacrylamide were from Bio-Rad Labs. [γ^{32}P]ATP (5,800 Ci/mmol) was prepared as previously described (12). All other standard chemicals were obtained commercially and were reagent grade quality.

Buffered solutions

Buffer A contained 500 mM sucrose, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 1 mM benzamidine, 0.5 mM TLCK, 1 μg/ml leupeptin. Buffer B contained 20 mM Tris-HCl (pH 8.5), 1 mM EDTA. Buffer C contained 5 mM sodium acetate (pH 5), 1 mM EDTA. Buffer D contained 20 mM imidazole (pH 7.5), 0.1 mM EDTA, 0.01% (w/v) Brij 35, 5% (v/v) glycerol. All these buffers contained, in addition, 1 mM PMSF and 0.5 mM DTT. Buffer E contained 40 mM Bistris (pH 7), 1 mg/ml bovine serum albumin, and 1 mM DTT.

Protein preparations

HMG-CoA reductase was purified as described previously (13). The catalytic subunits of protein phosphatases 1 and 2A were purified from rat liver by the method of Calvet, Gil, and Hegardt (3). Liver protein phosphatases were characterized by their capacity to dephosphorylate the α or β subunit of phosphorylase b kinase and by their sensitivity to inhibition by homogenous rabbit skeletal muscle inhibitor-2 (14). The catalytic subunits of protein phosphatase 1 and protein phosphatase 2A from rabbit skeletal muscle were purified by the method of Resink et al. (15). Inhibitor-2 was purified from rabbit skeletal muscle by the method of Yang, Vandenheede, and Merlevede (16). Crystalline rabbit skeletal muscle phosphorylase b was prepared as described by Fischer and Krebs (17). [^{32}P]Phosphorylase a was prepared from phosphorylase b and rabbit muscle phosphorylase kinase as previously described (18). Phosphorylase kinase was prepared as described by Cohen (19). Glycogen synthase kinase-3 was purified from rabbit skeletal muscle by the method of Cohen (20).

Assay methods

HMG-CoA reductase activity was determined as described by Bové and Hegardt (21). 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.

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 to a control without phosphatase as previously described (22). One unit of HMG-CoA reductase 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.

The purified protein inhibitor was tested for its capacity to inhibit HMG-CoA reductase phosphatase activity. Prior to the assay, protein inhibitor and protein phosphatase 1 were diluted with buffer E containing 6 mM $MnCl_2$. Then, a sample of inhibitor (0.015 ml) was preincubated for 10 min at 37°C with protein phosphatase 1 (0.015 ml, containing 2–4 U). The reaction was initiated with 0.02 ml of homogenous HMG-CoA reductase (0.22 μg) in buffer E. After 15 min at 37°C, the HMG-CoA reductase phosphatase activity was determined as described above. Assays were carried out in duplicate and controls were included in which inhibitor or protein phosphatase were omitted. One unit of the protein inhibitor decreased the activity of HMG-CoA reductase phosphatase 1 by 50% in the standard assay conditions.

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 (23). One unit of phosphorylase phosphatase activity is defined as the amount of enzyme that catalyzes the release of 1 nmol phosphate per min.

Inhibitor-2 from rabbit skeletal muscle was assayed by its ability to inhibit protein phosphatase 1 using [^{32}P]-phosphorylase as a substrate according to the method of Foulkes and Cohen (6). One unit of the inhibitor-2 was the amount that inhibited the phosphorylase phosphatase activity of protein phosphatase 1 by 50% under standard assay conditions.

Purification of rat liver inhibitor

The protocol for the purification of rat liver protein inhibitor comprising column chromatographies on DEAE-cellulose at pH 8.5 and pH 5, Bio-Gel A-0.5m and Blue Sepharose CL 6B has been reported in a previous study (12). In all the chromatographic procedures we assayed the inhibitory activity of the protein inhibitor towards type 1 protein phosphatase from rat liver, using HMG-CoA reductase as the substrate.

Gel electrophoresis

The purified protein inhibitor was analyzed by SDS-polyacrylamide slab gels (10%) according to the method of Laemmli (24). Gels were stained with 0.25% Coomassie Brilliant Blue. For the determination of molecular mass, standard molecular mass proteins obtained from Sigma were used.

Agarose gel electrophoresis

The purified protein inhibitor was also analyzed by SDS-agarose 4% gel electrophoresis (submarine type) as described by Sakakibara et al. (25). The finished gel was sliced in 3-mm pieces, homogenized in 0.5 ml Bistris 40 mM (pH 7), and centrifuged at 10,000 *g* for 10 min. The

supernatant solution was dialyzed for 12 h in the same buffer and the fractions were assayed for inhibitory activity towards HMG-CoA reductase phosphatase activity.

Protein determination

Protein was determined by the method of Bradford (26). Bovine serum albumin was used as the standard.

RESULTS

Purification of the rat liver protein inhibitor

Chromatography on DEAE-cellulose, pH 8.5, of the inhibitory activity towards HMG-CoA reductase phosphatase (type 1) is shown in **Fig. 1A**. A broad peak was located between 140 and 400 mM NaCl. When active inhibitory fractions were separated on DEAE-cellulose at pH 5 (Fig. 1B), two peaks with activity were seen, the major one located between salt concentrations of 200 and 350 mM NaCl. The final two steps of the purification involved gel filtration on Bio-Gel A-0.5m (**Fig. 2**) and Blue Sepharose CL 6B (**Fig. 3**). The gel filtration proved to be effective since the inhibitory activity was separated in a symmetric peak and emerged just before ovalbumin (Fig. 2, insert). In contrast, most of the contaminant proteins were eluted in two peaks, at higher and lower M_r positions. The last step was a Blue Sepharose CL 6B chroma-

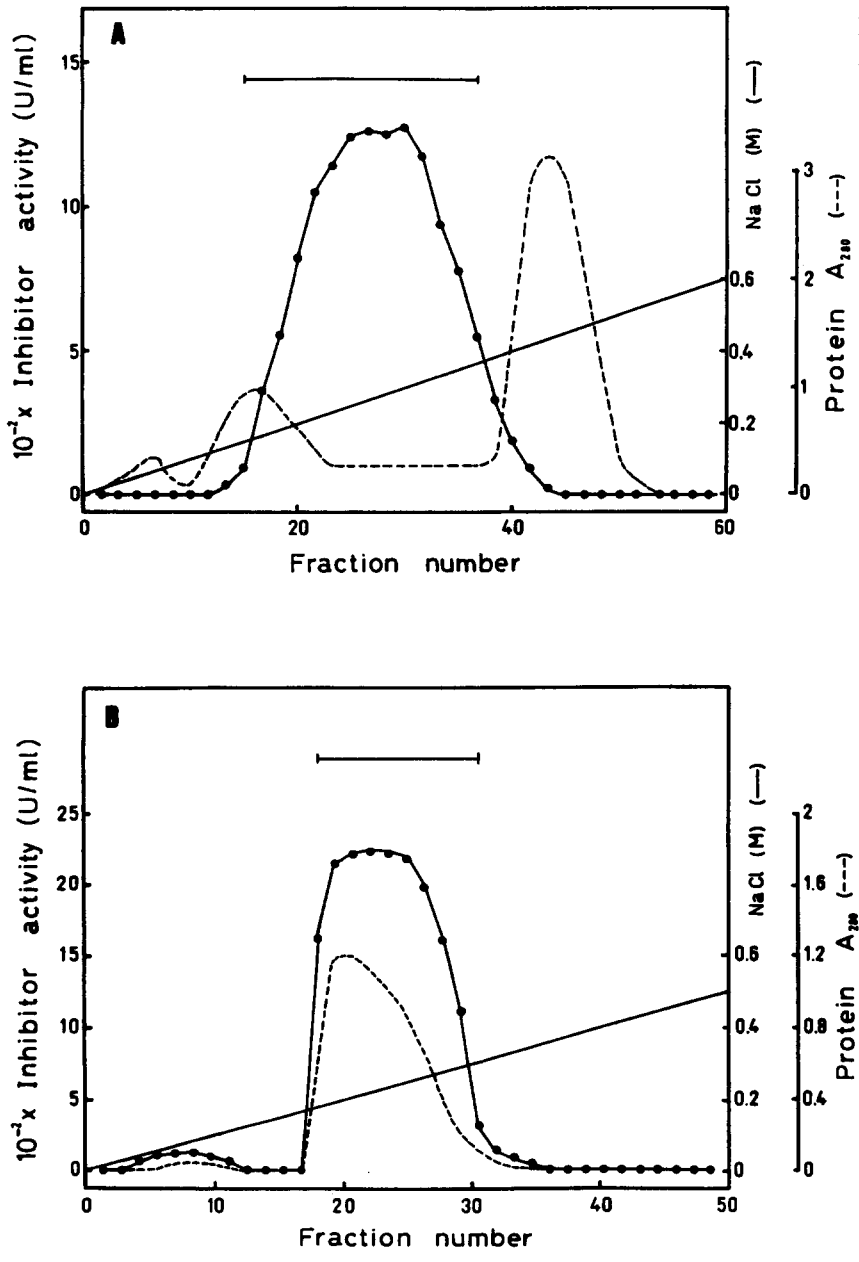

Fig. 1. A: Chromatography of rat liver protein inhibitor on DEAE-cellulose at pH 8.5; (●——●) inhibitor activity; (---) absorbance at 280 nm. Fractions 15 to 35, as shown by the bar, were pooled and precipitated. B: Chromatography of rat liver protein inhibitor on DEAE-cellulose at pH 5. Fractions 17 to 31, as shown by the bar, were collected.

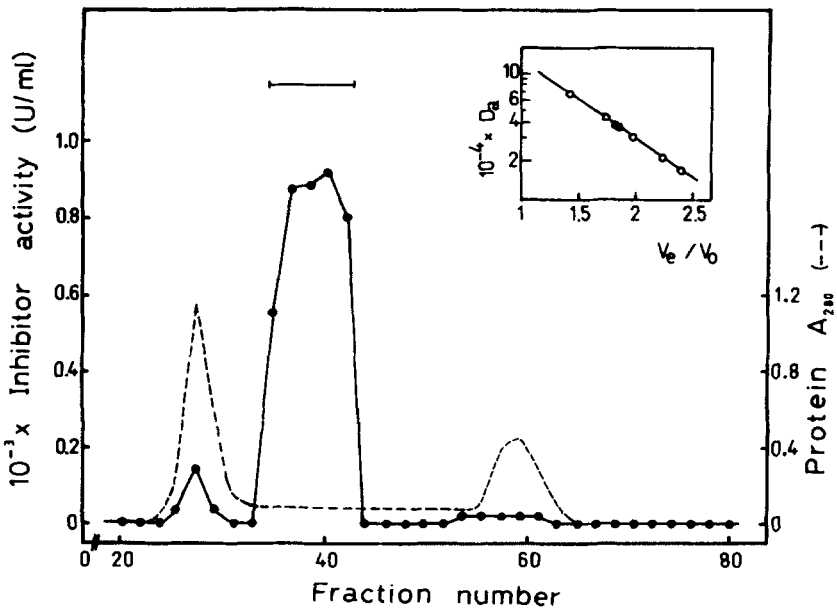

Fig. 2. Chromatography of rat liver protein inhibitor on Bio-Gel A-0.5 m; (●——●) inhibitor activity; (---) absorbance at 280 nm. Fractions 37 to 42, as shown by the bar, were pooled and diluted fourfold in buffer D. Molecular mass of inhibitor-2 was determined by gel filtration using this column (insert). Standard molecular weight proteins were: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa), and myoglobin (17 kDa). The marker proteins were located by absorbance measurements, while protein inhibitor was assayed as described in Materials and Methods.

tography in which most of the inhibitory activity eluted as a peak at 600 mM NaCl. A summary of the purification is given in **Table 1**. It can be seen that rat liver protein inhibitor was purified 4,000-fold with a yield of 4%. The activity of the purified protein was stable for at least 3 months when stored lyophilized at −20°C.

Gel electrophoresis

When the fractions of the last step were subjected to polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate, a single protein band was observed (**Fig. 4**). The mobility of the protein inhibitor corresponded to that of a protein of molecular mass of 31 kDa.

When the homogeneous preparations of the rat liver HMG-CoA reductase phosphatase inhibitor were electrophoresed in SDS-agarose gels and the gels were sliced and assayed for inhibitor activity using type 1 phosphatase and HMG-CoA reductase as substrate, the inhibitory activity was found to correspond to a protein of molecular mass of 31 kDa (**Fig. 5**).

Comparing this value with that reported in the literature for inhibitor-2 from all rat tissues as being 33 kDa (27), it is possible that a proteolytic process during the purification procedure removed a few amino acids in our preparation.

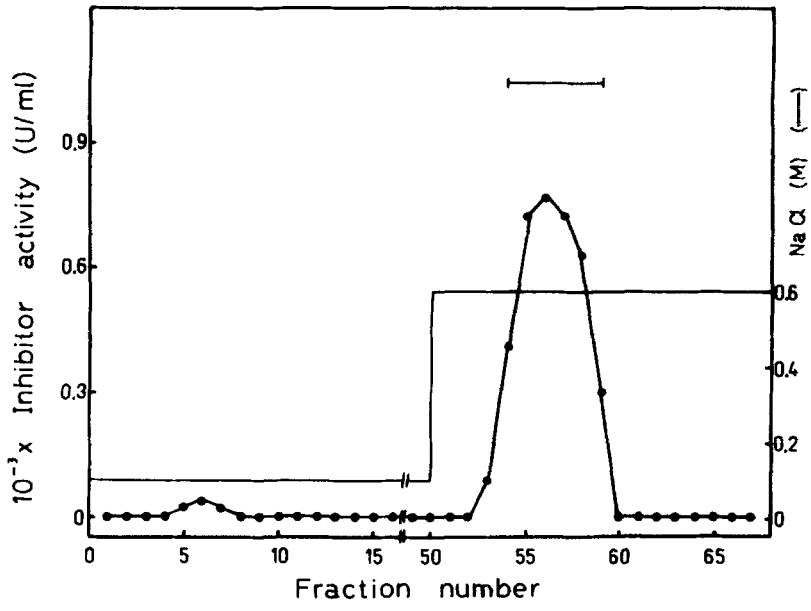

Fig. 3. Chromatography of rat liver protein inhibitor on Blue Sepharose CL 6B; (●——●) inhibitory activity. Elution began at fraction 50 with 0.6 M NaCl in buffer D. The active fractions were dialyzed, lyophilized, and stored at −20°C.

TABLE 1. Purification of the heat-stable protein inhibitor of phosphoprotein phosphatase from rat liver

Fraction	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	*units* × 10^{-3}	*units/mg*	*-fold*	*%*
Cytosol	6,900	260	37	1	100
Boiled supernatant	102	358	3,500	94	137
15% Trichloroacetic acid	85	300	3,580	94	110
DEAE-cellulose, pH 8.5	20	128	6,400	172	49
DEAE-cellulose, pH 5	11.2	80	7,140	192	30
Bio-Gel A-0.5m	1.8	23	12,700	729	8
Blue Sepharose CL 6B	0.08	12	150,000	4,000	4

Heat-stable protein inhibitor was purified as described in the text. The inhibitor activity in the liver cytosol was determined on the supernatant obtained after heating in a boiling water bath and then desalted on Sephadex G-25 in order to remove low-molecular mass protein phosphatase-inhibitory material. The amount of liver used was 115 g.

Effect of the protein inhibitor on protein phosphatases 1 and 2A and substrate HMG-CoA reductase

The rat liver inhibitor inhibits activity of HMG-CoA reductase phosphatase type 1 at nanomolar concentrations (**Fig. 6**). When purified phosphatase 2A was used instead of phosphatase 1, almost 300 times as much protein inhibitor was necessary to achieve the same inhibition. Similar inhibitions were obtained in the present study using a preparation of inhibitor-2 from rabbit skeletal muscle. When phosphorylase was used as substrate instead of HMG-CoA reductase, similar results were obtained (data not shown).

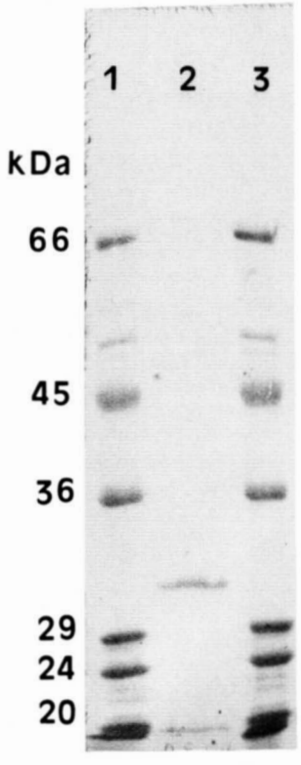

Fig. 4. SDS-polyacrylamide gel electrophoresis of rat liver protein inhibitor. The preparation from Blue Sepharose CL 6B was concentrated and a sample containing 2 μg was run on 10% slab gels (lane 2) according to Laemmli (24). The gel was stained with Coomassie blue. The two-digit numbers denote the positions (lanes 1 and 3) of the marker proteins bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and soybean trypsin inhibitor (20.5 kDa).

Phosphorylation of the protein inhibitor by protein kinases

The purified protein inhibitor is phosphorylated by the protein kinases. Two μg of the inhibitor was incubated with 1 mM magnesium acetate, 50 μM [γ^{32}P]ATP, and either 5 μg glycogen synthase kinase-3 or 6 mU cAMP-dependent protein kinase and cAMP in a total volume of 50 μl. The mixture was then analyzed by electrophoresis in SDS-PAGE and autoradiography; the phosphorylation of the inhibitor was clearly demonstrated (**Fig. 7**). The inhibitory activity was not changed by the phosphorylation process.

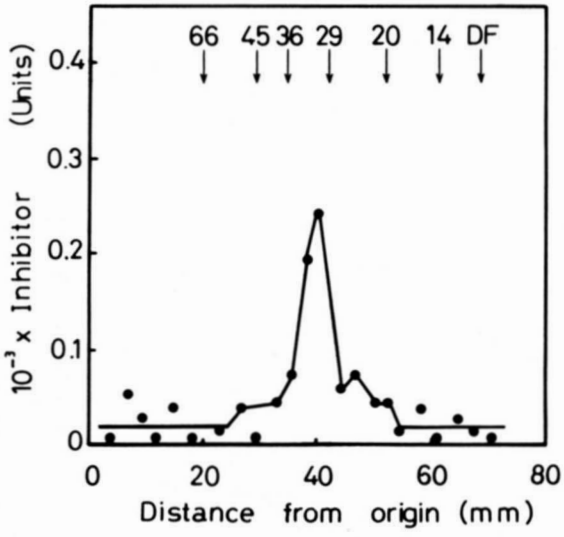

Fig. 5. SDS-agarose gel electrophoresis (submarine type) of inhibitor from rat liver. Ten μg of purified protein inhibitor was run on 4% agarose gel according to Sakakibara et al. (25). The gel was sliced and the 3-mm pieces were extracted, dialyzed in 40 mM Bistris (pH 7), and assayed for inhibitory activity as described above. The same marker proteins as shown in Fig. 4 were used.

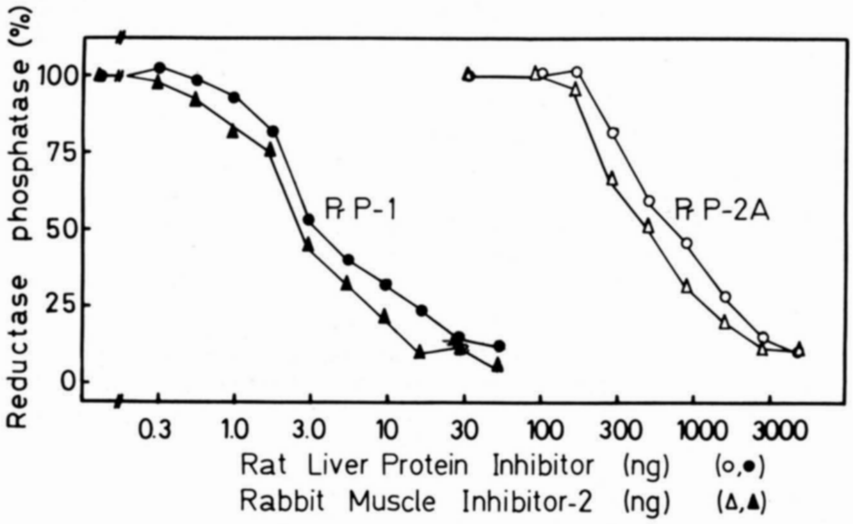

Fig. 6. Inhibition of type 1 and type 2 phosphatase catalytic proteins by rat liver protein inhibitor and rabbit skeletal muscle inhibitor-2. Purified type 1 (closed symbols) and type 2A (open symbols) phosphatases were incubated with the purified rat liver inhibitor (●, ○) and with rabbit skeletal muscle inhibitor-2 (▲, △) for 15 min at 37°C with a total volume of 30 μl. Inhibition of HMG-CoA reductase phosphatase activity of both type 1 and type 2 enzymes was measured and represented as percentage of the phosphatase activity without inhibitor.

DISCUSSION

This report represents the first study of the isolation of a homogeneous preparation of a protein inhibitor from rat liver that, at small concentrations, inhibits type 1 protein phosphatases using HMG-CoA reductase as substrate.

The procedure for the purification of the rat liver inhibitor described here basically combines the procedures used by others for the purification of liver inhibitors (10, 11) towards protein phosphatases, that is, DEAE-cellulose chromatography and gel filtration. In addition, it introduces a new step as the rat liver inhibitor was not electrophoretically pure. We used chromatography on Sepharose CL 6B, eluting the inhibitor with 0.6 M NaCl in buffer D. When we adopted the elution system used by Gruppuso et al. (28) for purification of muscle inhibitor, of 0.1 M NaCl in buffer D as the eluting agent, the inhibitor protein was not eluted but remained in the column. The reason for this discrepancy in the chromatographic properties is not yet clear, although it is probably due to the differences between the inhibitor from rat liver and that from rabbit skeletal muscle.

Taking into account that there is no previous report of the purification of an inhibitor towards type 1 HMG-CoA reductase phosphatase activity from rat liver, comparison

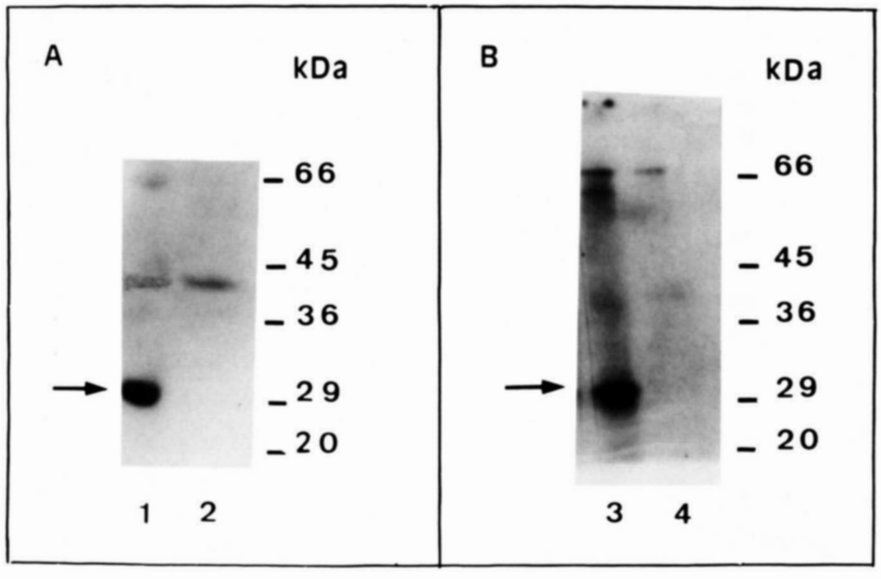

Fig. 7. Phosphorylation of the liver protein inhibitor by glycogen synthase kinase-3 and cAMP-dependent protein kinase. A: Autoradiography of the ^{32}P-phosphorylated protein inhibitor by cAMP-dependent protein kinase (lane 1). Lane 2 is a control without inhibitor. B: Autoradiography of the ^{32}P-phosphorylated protein inhibitor by glycogen synthase kinase-3 (lane 3). Lane 4 is a control without inhibitor. Arrows refer to the protein inhibitor. Molecular weight markers are shown to the right.

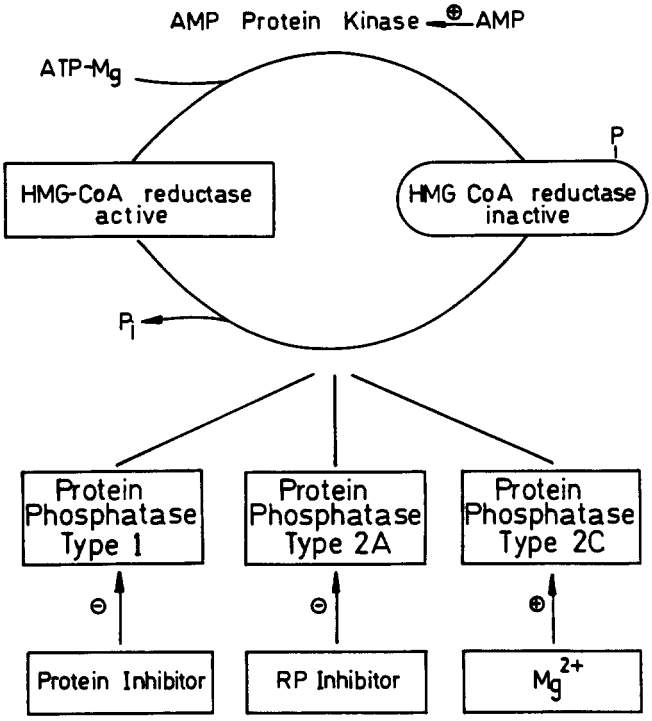

Fig. 8. Schematic representation of the modulation of the enzyme activity of HMG-CoA reductase and its dephosphorylating enzymes. RP Inhibitor (12) specifically inhibits type 2A phosphatase. Liver protein inhibitor inhibits type 1 protein phosphatase.

of molecular or kinetic properties of this inhibitor should be carried out with preparations from other organs or different animals. There is a high correspondence in the properties of the rat liver inhibitor with respect to the inhibitor-2 from either rabbit skeletal muscle or other tissues (29). Rat liver inhibitor and rabbit skeletal muscle inhibitor-2 have molecular masses of 31 kDa, and inhibit protein phosphatase 1 at nanomolar concentrations and protein phosphatase 2A at micromolar concentrations. Both also show analogous properties not only in their kinetic properties but, owing to the fact that both are phosphorylated by cAMP-dependent protein kinase and glycogen synthase kinase-3 without change in inhibitor activity, both are able to reconstitute the Mg-ATP-dependent protein phosphatase (data not shown).

Ingebritsen and Gibson reported in 1980 (30) that phosphatase inhibitor-2, partially purified from rat liver according to the method of Khandelwal and Zinman (10), inhibited phosphorylase a phosphatase activity identically, using either phosphorylase a or HMG-CoA reductase as substrates. At present we cannot exclude the possibility that their inhibitory preparation was a mixture of RP inhibitor and inhibitor-2, and that the phosphatase used in this experiment could be a mixture of the catalytic subunits of both type 1 and type 2 phosphatases.

Beg et al. (31–33) have postulated that inhibitor-1 and inhibitor-2 may modulate HMG-CoA reductase phosphatase activity as a system of controlling cholesterol biosynthesis. The speculation was based on the low specificity of protein phosphatases for different substrates. The inhibitory effect of inhibitor-1 and inhibitor-2 had been previously tested on protein phosphatases using phosphorylase a as substrate (34). The inhibitory effect of inhibitor-2 had been tested on protein phosphatases using glycogen synthase as substrate (35, 36), acetyl-CoA carboxylase (37), and the myosin P-light chain (38, 39). However, no data has been reported up to now on the inhibitory effect of purified inhibitor-1 and inhibitor-2 on HMG-CoA reductase phosphatase activity.

Taking into account that Cohen and co-workers (27, 40) have presented evidence that inhibitor-1 is not present in rat liver, the variation in the phosphorylation state of HMG-CoA reductase cannot underlie the action of this protein inhibitor.

Our current concept of the regulation by covalent modification of the enyzme activity of HMG-CoA reductase in rat liver is depicted in **Fig. 8**. The dephosphorylation process is achieved by protein phosphatases 1, 2A, or 2C. Nothing is known at present about the regulation of type 2C phosphatase activity by inhibitory proteins. The modulation of the other two phosphatase activities is carried out by two different protein inhibitors, RP-inhibitor and the protein inhibitor presented here. Protein phosphatase 2A is inhibited by RP inhibitor (12) in a rather specific way and, furthermore, protein phosphatase 1 from rat liver is solely inhibited by the liver inhibitor that shows characteristics similar to the rabbit skeletal muscle inhibitor-2.

The occurrence of two different inhibitors specifically inhibiting either type 2A or type 1 protein phosphatases shows the accuracy of the regulation of cholesterol biosynthesis through the dephosphorylation of its enzyme regulator HMG-CoA reductase.

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