Inactivation of rat liver HMG-CoA reductase phosphatases by polycarboxylic acids

Fausto G. Hegardt, Gregorio Gil,[1] and Victoria E. Calvet

Department of Biochemistry, University of Barcelona School of Pharmacy, Barcelona-28, Spain

Abstract Incubation of the four purified HMG-CoA reductase phosphatases with the sodium salts of eleven polycarboxylic acids at concentrations of 40 mM, inactivated the enzymes to different degrees depending on the structure of the carboxylic acids. Maleate, malonate, oxalate, citrate, and hydroxymethylglutarate produced full inactivation at the concentration tested. When the four phosphatases were incubated with these acids, a concentration-dependent inactivation was observed. Fumarate, the *trans* isomer of maleate, produced little inactivation of the four phosphatases. Mevalonate did not inactivate at all. A relationship between those concentrations of acid that produced a 50% inactivation and the logarithm of the stability constant of Mg^{2+} or Mn^{2+} salts of polycarboxylic acids was observed. When reductase phosphatases were incubated with mixtures of polycarboxylic sodium salts and Mg^{2+} or Mn^{2+}, an increase in the molar ratio divalent cation/carboxylic acid determined an increase in the four reductase phosphatase activities. The inactivating effect of citrate was on the phosphatases (high and low forms) and not on the substrates (MHG-CoA reductase, phosphorylase, and glycogen synthase). Reactivation of the citrate-inactivated phosphatases by Mn^{2+} and Mg^{2+} depended on the phosphorylated substrates, Mn^{2+} being the better activator. It is concluded that HMG-CoA reductase phosphatases are metalloenzymes.—**Hegardt, F. G., G. Gil, and V. E. Calvet.** Inactivation of rat liver HMG-CoA reductase phosphatases by polycarboxylic acids. *J. Lipid Res.* 1983. **24:** 821–830.

Supplementary key words cholesterol biosynthesis • HMG-CoA reductase phosphatases • dicarboxylic acids • citrate • divalent cations • metalloenzymes • phosphorylase • glycogen synthase

3-Hydroxy-3-methylglutaryl coenzyme A reductase (mevalonate: $NADP^+$ oxidoreductase (CoA-acylating) E.C. 1.1.1.34) appears to control the rate of cholesterol synthesis in the liver of a number of vertebrates (1, 2). The mechanisms for the changes in HMG-CoA reductase activity have received much attention. Recent studies support the notion that the activity of reductase may be regulated by enzyme modification. Microsomal (3–6) and solubilized (7) reductase is inactivated in vitro in the presence of Mg-ATP and a reductase kinase with the covalent binding of phosphate in two sites or sets of sites to the reductase molecule (8). Inactivated homogeneous ^{32}P-labeled HMG-CoA reductase is reactivated by rat liver HMG-CoA reductase phosphatases with a concomitant release of ^{32}P (9).

In another paper (10), we reported the inhibitory effect of phosphate and pyrophosphate on HMG-CoA reductase phosphatases isolated by us (9), and the reversal of this effect by divalent cations. Moreover, the direct influence of Mg^{2+} and Mn^{2+} as activators of phosphatases was studied. The activation by divalent cations and inactivation by their chelators, suggested to us that reductase phosphatases could be metalloenzymes with a metal bound at the catalytic site.

Several studies on Pi, ATP, and divalent cation regulation of phosphoprotein phosphatases have been reported. First, Kato, Kobayashi, and Sato (11) and then Burchell and Cohen (12) suggested that rabbit muscle phosphorylase phosphatase might be a metalloenzyme as it was inactivated by phosphate and reactivated by Mn^{2+}. Similar arguments were given by Khatra and Soderling (13) and Yan and Graves (14). However, Brautigan, Picton, and Fischer (15) could not show the incorporation of ^{54}Mn into phosphorylase phosphatase and Khandelwal and Kamani (16) could not show the incorporation of [γ-^{32}P]ATP into the enzyme. Therefore, we considered it of interest to contribute more evidence to the resolution of this topic.

In this study, we show that several polycarboxylic acids inhibit HMG-CoA reductase phosphatases from rat liver. We present evidence of the close relationship between the chelating capacity of these carboxylic acids on divalent cations (expressed by the stability constant of the complexes) and their inhibition of reductase phosphatases. Moreover, we can show the gradual reversal of the inhibitory effect when, prior to the assay, acids were mixed with divalent cations. In addition, we prove that the inhibition of HMG-CoA reductase phosphatase

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; reductase, HMG-CoA reductase; MVL, mevalonolactone; HMG-Ac, hydroxymethylglutaric acid; PMSF, phenylmethylsulfonyl fluoride.

[1] Present address: Department of Molecular Genetics, The University of Texas Health Science Center at Dallas, Dallas, TX 75235.

by polycarboxylic acids is due to the interaction of these acids with the phosphatase and not with the substrate, HMG-CoA reductase. We demonstrate that citrate inhibits the four reductase phosphatase activities toward two different phosphoproteins other than HMG-CoA reductase, namely, phosphorylase a and glycogen synthase D. We concluded from these data that HMG-CoA reductase phosphatases are metalloenzymes, and that Mn^{2+} is probably the metal ion bound to the enzyme.

MATERIALS AND METHODS

Chemicals

Most of the reagents used in this study have been reported previously (9). Manganese chloride, magnesium chloride, tartaric, maleic, and citric acids were from Merck. Succinic, malic, fumaric, malonic, hydroxymethylglutaric, α-ketoglutaric, and oxaloacetic acids and α-amylase were from Sigma. Oxalic acid was from Carlo Erba.

Buffer solutions

Buffer A was 100 mM sucrose, 40 mM potassium phosphate, 10 mM sodium pyrophosphate, 30 mM EDTA, 50 mM KCl, and 1 mM dithiothreitol, pH 7.2. Buffer B contained 40 mM Bis-Tris, 1 mM dithiothreitol, 300 mM sucrose, and 0.05% w/v serum albumin, pH 6.5. Buffer C contained 50 mM Tris, 5 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM PMSF, 1 mM dithiothreitol, and 50 mM NaF, pH 7.4. Buffer D contained 10 mM glycylglycyne, 5 mM dithiothreitol, and 0.4 mg/ml serum albumin, pH 7.4.

Purification of the enzymes

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 (17), the enzyme was inactivated with 4 mM ATP and purified to homogeneity by affinity chromatography on Affi-Gel Blue and HMG-CoA Agarose (7). Under these conditions, the fraction of HMG-CoA reductase that remained in the active form was 13% (996 units/mg protein). This partially inactivated HMG-CoA reductase preparation will be referred to in the text as "homogeneous HMG-CoA reductase" and was the substrate for the phosphatases.

HMG-CoA reductase phosphatases were purified as described previously (8). Reductase phosphatases Ia and IIa are the high molecular weight enzymes (480,000 and 310,000, respectively) appearing in positions 1 and 2 in the DEAE-cellulose chromatography. Reductase phosphatases Ib and IIb are the low molecular weight forms (35,000) obtained from reductase phosphatases Ia and IIa after ethanol treatment at room temperature. Specific activities were 310, 710, 11320, and 21480 units/mg protein, respectively, for phosphatases Ia, IIa, Ib, and IIb. The four reductase phosphatases removed ^{32}P from homogeneous ^{32}P-labeled HMG-CoA reductase (5–7). In addition, the reductase phosphatases exhibited protein phosphatase activities towards homogeneous rabbit muscle phosphorylase a and homogeneous rat liver glycogen synthase D.

Rabbit muscle phosphorylase b was purified to homogeneity according to the method of Fischer and Krebs (18). Rabbit skeletal muscle phosphorylase kinase was purified by the method of Cohen (19).

Rabbit skeletal muscle ^{32}P phosphorylase a was prepared from phosphorylase b using [γ-^{32}P]ATP and phosphorylase kinase as described by Krebs, Kent, and Fischer (20). The ^{32}P phosphorylase contained 3.44 mol of bound ^{32}P per tetramer of phosphorylase.

Rat liver glycogen synthase D was purified essentially as described by Hiraga et al. (21). In brief, washed glycogen granules from fresh rat liver were treated with human α-amylase for 45 min and applied to a DEAE cellulose column (2.3 × 12 cm) previously equilibrated with buffer C. The column was washed with buffer C and the enzyme activity was eluted by applying a linear 0.005 to 0.5 M NaCl gradient in 200 ml of buffer C. Fractions containing glycogen synthase activity were pooled, mixed with glycogen (0.5 mg/ml), precipitated with 15% ethanol at −6°C, and centrifuged at 15,000 *g* for 30 min at −10°C. The particulate fraction containing the enzyme was resuspended in buffer D and dialyzed against the same buffer.

Enzymatic assays

HMG-CoA reductase activity was assayed as previously described (9). HMG-CoA reductase phosphatase activity was determined by the increase in HMG-CoA reductase activity of the inactivated homogeneous enzyme over the control without phosphatase; the assay was conducted as follows. Samples of the phosphatases to be assayed (10 μl, 20 units) were mixed with homogeneous HMG-CoA reductase (5 μl, 0.22 μg) and 10 μl of buffer B with or without effector and incubated at 37°C. After 15 min the reaction was stopped by adding 35 μl of buffer A. Aliquots of 50 μl of this mixture were incubated at 37°C for 30 min with 55 μl of a solution containing 22 μmol of Tris, 0.5 units of glucose-6-phosphate dehydrogenase, 5 μmol of EDTA, 0.4 μmol of dithiothreitol, 5 μmol of glucose-6-phosphate, 0.3 μmol of $NADP^+$, and 0.05 mg of serum albumin. This last incubation eliminated the unlabeled HMG-CoA, which accompanied purified HMG-CoA reductase (7), by transforming it into mevalonate, the product of

the enzymic reaction. The reaction was started by addition of 5 μl (11.2 μg) of ^{3}H-labeled HMG-CoA (22,500 dpm/nmol) and further incubation at 50°C for 10 min to convert mevalonate to mevalonolactone. Separation of [^{3}H]mevalonolactone and [^{3}H]hydroxymethylglutaric was performed by the "mixed phase" method proposed by Philipp and Shapiro (22). The amounts of reductase phosphatase assayed were such that less than 15% of the substrate was activated in a 15-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 each case to determine whether effectors react with the substrate HMG-CoA reductase.

Phosphorylase phosphatase activity was determined by the release of ^{32}P from ^{32}P-labeled phosphorylase a (0.5 mg/ml) at 30°C as described by Khandelwal, Vandenheede, and Krebs (23). Glycogen synthase activity was determined by the formation of synthase I from synthase D essentially as described by Hiraga et al. (21). The standard assay mixture (0.05 ml) was buffer D to which was added 0.008 units of glycogen synthase D and the solution containing the phosphatase. After incubation for 20 min at 30°C, the 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 (24). Total synthase (I + D) activity was determined in the presence of 10 mM glucose-6-phosphate.

Units

One unit of HMG-CoA reductase was defined as the amount of enzyme that converts 1 nmol of HMG-CoA into mevalonate per min. One unit of HMG-CoA reductase phosphatase was defined as the activity of enzyme that increases the activity of HMG-CoA reductase 1 munit per min. 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 one munit the glycogen synthase activity per min. One unit of phosphorylase phosphatase is the amount of enzyme that catalyzes the release of 1 nmol phosphate in 1 min.

RESULTS

Inactivation of rat liver HMG-CoA reductase phosphatases by polycarboxylic acids

The effects of the sodium salts of citric acid and ten dicarboxylic acids on purified HMG-CoA reductase phosphatases Ia and IIa (high Mr forms) and Ib and IIb (low Mr forms) (9) were examined. As shown in **Table 1,** maleate, malonate, oxalate, citrate, and 3-hydroxy-3-methylglutarate at 40 mM concentration fully inactivated the four reductase phosphatases. The other dicarboxylates produced variable inactivation depending on their structure. In general, the longer chain acids of the same series produced less inactivation. The following sequence of inactivation could be observed: oxalic > malonic > succinic, and oxaloacetic > α-ketoglutaric. We did not observe any inactivation of HMG-CoA reductase by dicarboxylic acids. The behavior of those dicarboxylic acids that produced full inactivation was investigated in detail.

TABLE 1. Effect of polycarboxylic acids on the activities of purified rat liver HMG-CoA reductase phosphatases

Additions	Reductase Phosphatase Ia	Reductase Phosphatase IIa	Reductase Phosphatase Ib	Reductase Phosphatase IIb
None	100	100	100	100
Succinate	105	120	70	84
α-Ketoglutarate	94	101	47	37
Tartrate	81	117	81	85
Oxaloacetate	32	54	26	32
Malate	35	80	55	34
Fumarate	80	100	60	70
Maleate	0	0	0	0
Malonate	0	0	0	0
Oxalate	0	0	0	0
Citrate	0	0	0	0
Hydroxymethylglutarate	0	0	0	0

Purified HMG-CoA reductase phosphatases Ia, IIa, Ib, and IIb, 20 units each, were incubated with partially inactivated homogeneous HMG-CoA reductase (0.22 μg) and different carboxylic acids (as sodium salts) at concentrations of 40 mM, in a total volume of 25 μl. Aliquots were withdrawn, diluted, and the HMG-CoA reductase activity was determined. Phosphatase activity without added acids was taken as 100%. Figures represent means of three experiments.

Effect of malonate and oxalate

When the four reductase phosphatases Ia, IIa, Ib, and IIb were incubated with HMG-CoA reductase and increasing amounts of malonate, a concentration-dependent inactivation was observed (**Fig. 1**). A similar pattern of inactivation was found for the four phosphatases. The concentrations that produced 50% inactivation (I_{50}) were about 3 mM for all phosphatases. In fact, 40 mM malonate completely inactivated reductase phosphatases. Oxalate produced a similar pattern of inactivation. The I_{50} for oxalate was between 1–2 mM, and full inactivation was produced at 5 mM.

Effect of maleate and fumarate

Under incubation conditions similar to those for malonate, maleate produced a concentration-dependent inactivation of the four reductase phosphatases. I_{50} values of about 5 mM were obtained; total inactivation of phosphatases occurred at 40 mM maleate (**Fig. 2**). The effect of the *trans* isomer of maleate was completely different. Fumarate at 10 mM had no effect on reductase phosphatases Ia, IIa, and Ib; the inhibition in all cases was less than 50% at concentrations of 40 mM. The structural difference between fumarate and maleate, consisting solely of the different spatial conformation of the two carboxylate groups, makes the stability constant of both Mg-fumarate and Mn-fumarate much lower than that of Mg-maleate or Mn-maleate, respectively. These data suggest that the affinity of the protein-bound cation and dicarboxylic acid determines the level of activity of reductase phosphatases.

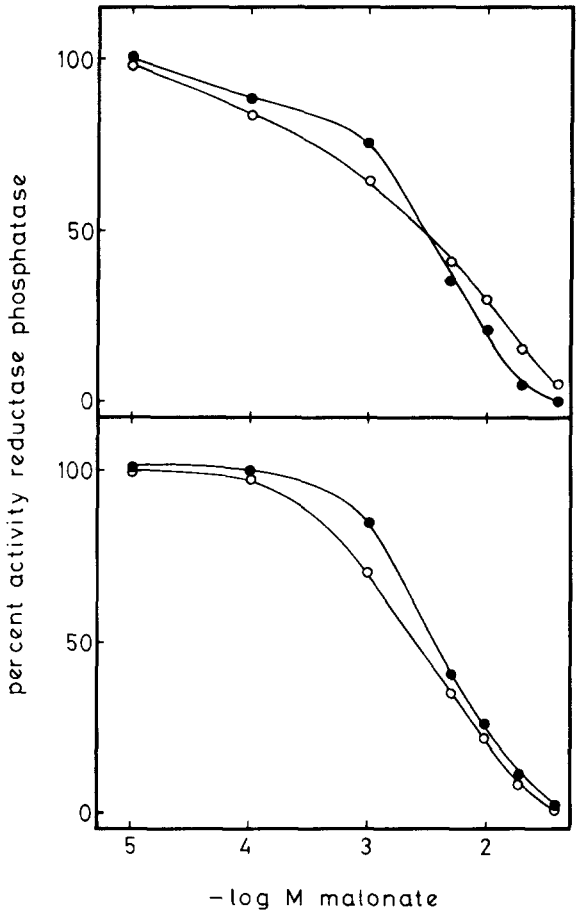

Fig. 1. Inactivation of purified rat liver HMG-CoA reductase phosphatases by different concentrations of malonate. Reductase phosphatases were incubated with partially inactivated homogeneous HMG-CoA reductase at 37°C with different concentrations of malonate. After 15 min incubation, aliguots were withdrawn, appropriately diluted with buffer A, and assayed for HMG-CoA reductase. Reductase phosphatase without added malonate was taken as 100%. Upper panel: high molecular weight reductase phosphatases Ia (●) and IIa (○). Lower panel: low molecular weight reductase phosphatases Ib (●) and IIb (○).

Effect of hydroxymethylglutarate and mevalonolactone

The study of the inactivation by 3-hydroxy-3-methylglutaric acid is of interest, since its CoA derivative is the substrate of HMG-CoA reductase. When 3-hydroxy-3-methylglutarate was incubated with HMG-CoA reductase phosphatases and HMG-CoA reductase, there was a pattern of inactivation of the phosphatases similar to that of maleate or malonate. Values of I_{50} for the phosphatases Ia, IIa, Ib, and IIb were 2, 10, 5, and 8 mM hydroxymethylglutarate, respectively. In a similar manner, 40 mM salt produced a complete inactivation of reductase phosphatases. In contrast, as shown in **Fig. 3,** mevalonolactone, a cyclic ester of the reduced hydroxymethylglutarate had no effect at all. It seems clear that the dicarboxylic structure is critical for inactivation of reductase phosphatases. These results suggest once more that chelation by the dicarboxylate of a metal ion bound to protein is probably responsible for the decrease in reductase phosphatase activity. Parallel experiments in which phosphatases were ommitted showed that neither 3-hydroxy-3-methylglutarate nor mevalonolactone inhibited HMG-CoA reductase at the concentrations used.

Relationship between I_{50} values and stability constants of Mg- and Mn-polycarboxylates

Carboxylic acids are chelators of divalent cations. The complex formed between the ligand and the metal ion is in equilibrium with the reactants according to the expression

$$\text{Carboxylate}^{2-} + \text{Metal}^{2+} \rightleftarrows \text{Me-Carboxylate}.$$

The stability constant is defined by the equation

$$K = \frac{[\text{Me-Carboxylate}]}{[\text{Carboxylate}^{2-}][\text{Me}^{2+}]}.$$

We thought it of interest to correlate the values of log K for the different Mg and Mn salts of maleic, malonic,

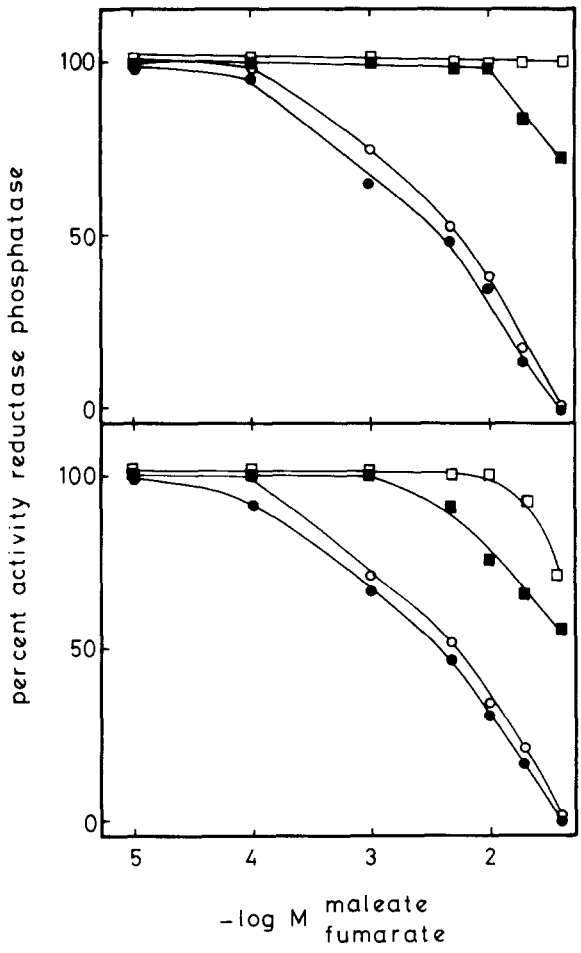

Fig. 2. Inactivation of purified rat liver HMG-CoA reductase phosphatases by different concentrations of maleate (●, ○) and fumarate (■, □). Reductase phosphatase activities without added acids were taken as 100%. Assay methods as in Fig. 1. Upper panel: reductase phosphatases Ia (●, ■) and IIa (○, □). Lower panel: Reductase phosphatases Ib (●, ■) and IIb (○, □).

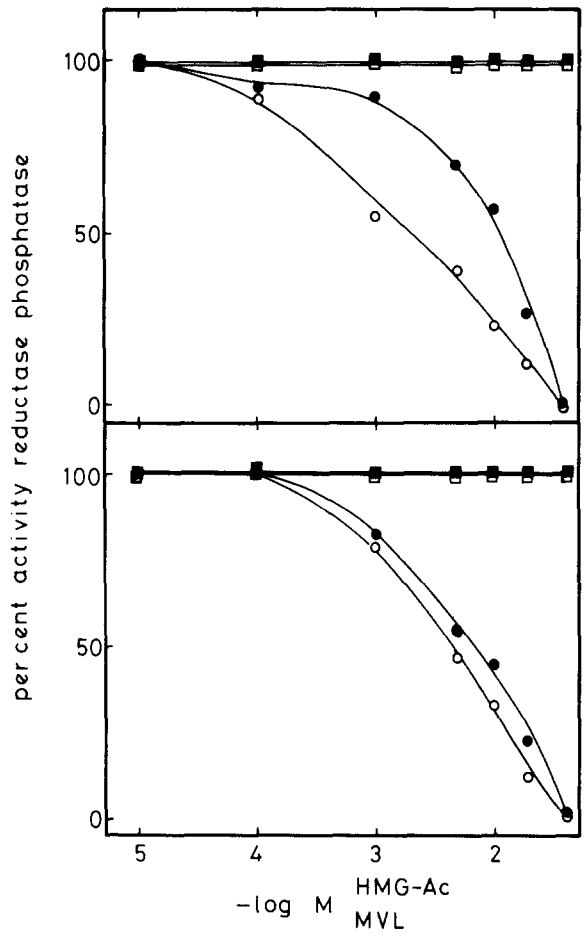

Fig. 3. Inactivation of purified rat liver HMG-CoA reductase phosphatases by different concentrations of hydroxymethylglutaric acid (●, ○) and mevalonolactone (■, □). Reductase phosphatase activities without added acids were taken as 100%. Assay methods as in Fig. 1 and symbols of reductase phosphatases as in Fig. 2.

oxalic, and citric acids (25–28) with the I_{50} value of the acids. The I_{50} value for citrate, 0.1–0.4 mM, depending on phosphatases, was taken from a study by Gil and Hegardt (29). Results appear in **Fig. 4.** The higher the value of log K, the lower the I_{50} value of the carboxylate. All reductase phosphatases behaved in a similar manner. This correlation appears to hold whether the divalent cation is Mn^{2+} or Mg^{2+}. It is seen in Fig. 4 that the log K values of the Mn^{2+}-complexes are associated with higher I_{50} values of the acids than are the log K values of the Mg^{2+}-complexes. This correlation suggests that if either Mg^{2+} or Mn^{2+} were to bind to the protein, Mn^{2+} would be more strongly bound than Mg^{2+}, as higher concentrations of carboxylate are necessary to produce the same I_{50}, at the same stability constant value.

The close correlation between logarithm of the stability constants and I_{50} concentrations strengthens the view that reductase phosphatases might be metalloen-

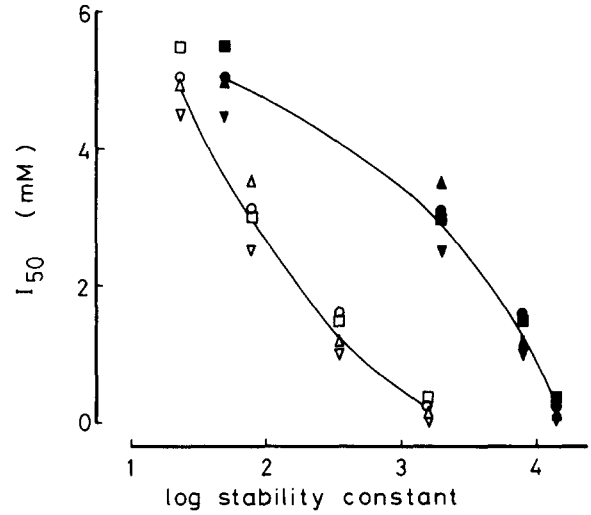

Fig. 4. Correlation between the logarithm of stability constants of Mg (open symbols) and Mn (closed symbols) salts of different carboxylic acids and the concentration of acid that produces 50% inactivation (I_{50}) on reductase phosphatases Ia (●, ○) IIa (■, □) Ib (▲, △) and IIb (▼, ▽). Values of logarithm stability constants of the Mg and Mn salts of maleic (1.3, 1.7), malonic (1.9, 3.3), oxalic (2.55, 3.9), and citric (3.2, 4.1) acids have been obtained from references 25–28.

zymes. Mg or Mn salts of fumaric acid whose log K is about 0 do not produce significant inhibition. Mevalonolactone, having lost its dicarboxylic character as compared to hydroxymethylglutarate, is not a chelator and produces no inhibition.

Effect of divalent cation-polycarboxylate mixtures on the HMG-CoA reductase phosphatase activities

We have shown in another paper (10) that divalent cations activate HMG-CoA reductase phosphatase, both before and after inactivation with PPi. We tested the effects of combinations of divalent cations and polycarboxylic acids in HMG-CoA reductase phosphatase preparations. Concentrations of polycarboxylates (as sodium salts) were in all cases 10 mM, while divalent cation concentrations were varied to produce divalent cation/polycarboxylic acid molar ratios of 0, 0.25, 0.50, 0.75, and 1.0.

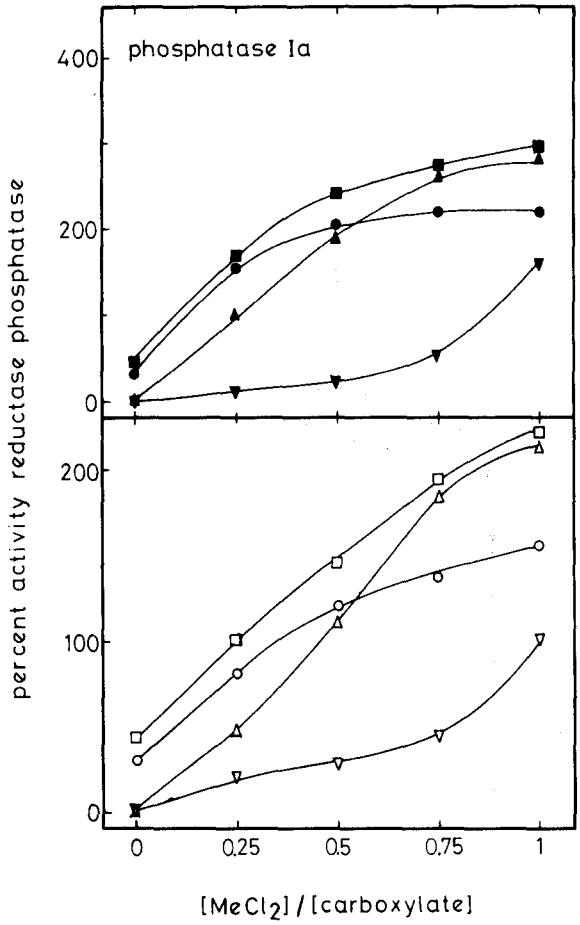

Fig. 5. Effect of varying molar ratios divalent cation/carboxylic acid on the activity of HMG-CoA reductase phosphatase Ia. Reductase phosphatase Ia (20 units) was incubated with homogeneous HMG-CoA reductase at pH 6.5 for 15 min at 37°C with mixtures of 10 mM carboxylic acid and increasing concentrations of divalent cation. Aliquots were withdrawn, adequately diluted, and assayed for HMG-CoA reductase. Reductase phosphatase Ia activity without added Mg^{2+} or Mn^{2+} was taken as 100%. Upper panel: divalent cation is Mn^{2+} (closed symbols). Lower panel: divalent cation is Mg^{2+} (open symbols). Symbols are: citric acid (▼, ▽), oxalic acid (▲, △), malonic acid (●, ○), and maleic acid (■, □).

Fig. 5 shows results of the correlation between these molar ratios and activation of reductase phosphatase Ia. An analogous pattern of curves was obtained for phosphatases IIa and Ib. Reductase phosphatase IIb cannot be completely reactivated even at a divalent cation/carboxylic acid ratio of 1.0. Several conclusions can be deduced from Fig. 5. *1*) Activation of HMG-CoA reductase phosphatase corresponds to increasing concentration of the divalent cation in the mixture. *2*) The order of the ascending portions of the curve is the same for the four phosphatases, i.e., citrate, oxalate, malonate, and maleate; this sequence is the same as the order of the values of log stability constants for both Mg and Mn salts. The curve corresponding to oxalate is anomalous, crossing the others at high ratios as if, at these high ratios, greater amounts of cation would be free in solution. We observed that mixtures of Mg^{2+} or Mn^{2+} ions with oxalate formed fine precipitates. *3*) Activation with Mn^{2+} was generally higher than with Mg^{2+} for every salt, suggesting that Mn^{2+} could be the divalent cation bound to the enzyme.

Table 2 reports the values of the molar ratios of Me^{2+}/acid mixtures at which the inhibitory effects of the four acids were abolished and at which the activities of the phosphatases were the same as observed in the controls and in absence of added cation. Two conclusions can be drawn from Table 2. *1*) For every reductase phosphatase and carboxylic acid, lower concentrations of Mn^{2+} than of Mg^{2+} were needed to abolish the inhibitory effects of the acids. *2*) for each reductase phosphatase and Mg^{2+} or Mn^{2+} ion, the increasing values of the cation/acid ratios from maleate to citrate at which the inhibitory effects of the acids are abolished, are in the same order as the increasing values of the stability constants of the cation-carboxylate complexes.

Reactivation of citrate-inactivated phosphatases by Mg^{2+} and Mn^{2+} using HMG-CoA reductase, glycogen phosphorylase, and glycogen synthase as substrates

It is well known that a given effector may affect a phosphoprotein phosphatase reaction by interacting with *1*) the phosphatase (16), *2*) the phosphoprotein substrate (30), and *3*) the phosphatase and the phosphoprotein substrate. In order to exclude the possibility that the inhibition caused by citrate and other carboxylic acids was a substrate-directed effect, we carried out some experiments involving: *i*) preincubation of HMG-CoA reductase phosphatases with citrate, *ii*) removing citrate from the phosphatase by extensive dialysis, and

TABLE 2. Effect of divalent cation/polycarboxylic acid mixtures on the activities of purified rat liver HMG-CoA reductase phosphatases

Reductase Phosphatase	Maleate		Malonate		Oxalate		Citrate	
	Mn	Mg	Mn	Mg	Mn	Mg	Mn	Mg
IIa	0.11	0.17	0.16	0.20	0.22	0.30	0.87	0.92
Ia	0.12	0.25	0.17	0.35	0.25	0.47	0.88	1.00
Ib	0.16	0.32	0.20	0.42	0.50	0.48	1.00	1.02

Purified HMG-CoA reductase phosphatases Ia, IIa, Ib, and IIb, 20 units each, were incubated at 37°C for 15 min (pH 6.5) with partially inactivated homogeneous HMG-CoA reductase (0.22 μg) and different mixtures of divalent cations and carboxylic acids, and HMG-CoA reductase activity was determined. The values of molar ratios divalent cation/carboxylic acids that produce 100% activity of reductase phosphatases IIa, Ia, and Ib are shown. Reductase phosphatase IIb did not reach 100% activity when molar ratios were lower than 1.

iii) assaying the phosphatase activity toward HMG-CoA reductase in the absence or presence of Mn^{2+} and Mg^{2+}. In addition, in order to demonstrate that the carboxylic acids and divalent cations affect the phosphatase and not the substrate, citrate-inactivated phosphatase activity was demonstrated on two different phosphoprotein substrates, glycogen synthase and phosphorylase, in the absence and presence of Mn^{2+} and Mg^{2+}.

Results appear in **Table 3.** It can be concluded that when citrate is removed by dialysis from the preincubation mixture, protein phosphatase activity remains completely inactivated towards its own substrate, HMG-CoA reductase, and toward other phosphorylated enzymes, such as phosphorylase a and glycogen synthase D. In addition, it is shown that reactivation of high molecular weight reductase phosphatases Ia and IIa with Mn^{2+} produces levels of activity towards the three substrates of the same order of magnitude. By contrast, Mg^{2+} does not reactivate the four reductase phosphatases at all when the substrate is glycogen phosphorylase or glycogen synthase. In addition, the four reductase phosphatases (substrate: HMG-CoA reductase) are fully reactivated by Mn^{2+} and by Mg^{2+}, with the exception of phosphatase IIb, which is only slightly reactivated by Mg^{2+}.

DISCUSSION

In the previous paper (10), we suggested the metalloenzymic character of reductase phosphatases on the basis of their activation by Mn^{2+} and Mg^{2+}, and inactivation by chelators of these ions, Pi and PPi. Several authors came to similar conclusions for phosphorylase phosphatase of various origins (13, 31, 32).

This study strengthens the idea that reductase phosphatase from rat liver needs a divalent cation for activity. Polycarboxylic acids that chelate these ions inhibit the reductase phosphatase. There is a close correlation between concentrations of ligand that produce 50% inactivation of reductase phosphatase and the log stability constant of Mg or Mn polycarboxylate complexes. This constant depends in turn of the structure of the acid. The comparison between the behavior of the four phosphatases with the *cis-trans* isomers maleate-fumarate is

TABLE 3. Reactivation of citrate-inactivated HMG-CoA reductase phosphatases by divalent cations

	Phosphatase Ia		Phosphatase IIa		Phosphatase Ib		Phosphatase IIb	
	Mn	Mg	Mn	Mg	Mn	Mg	Mn	Mg
HMG-CoA reductase	103	101	123	124	171	149	135	17
Phosphorylase	153	0	118	0	0	0	0	0
Glycogen synthase	151	0	84	0	52	0	0	0

Purified HMG-CoA reductase phosphatases Ia, IIa, Ib, and IIb, 20 units each, were independently incubated with 40 mM citrate. The solutions were extensively dialyzed and then incubated with either 5 mM Mn^{2+} or 5 mM Mg^{2+} and three purified phosphoprotein substrates, HMG-CoA reductase (0.22 μg), ^{32}P-labeled phosphorylase a (20 μg), and glycogen synthase D (0.008 units) for 15 min at 30°C and thereafter assayed for the three enzymatic activities. Enzymatic activities of the reductase phosphatases without any treatment, and after citrate preincubation and dialysis, had 100% and 0% activity, respectively.

significant. The *trans* isomer, in which the carboxylate groups are antiperiplanar and thus incapable of chelating Mn^{2+} or Mg^{2+} by intramolecular forces, caused very little inactivation. On the other hand, maleate produces an inactivation similar to other polycarboxylic acids whose carboxylate groups are at a similar spatial distance.

Hydroxymethylglutaric acid and mevalonic acid (the more stable form of mevalonolactone at pH 6.5) represent another significant example. Mevalonate is produced in the cell by a double reduction from the CoA ester of hydroxymethylglutaric acid, with formation of a primary alcohol. As a result, there is no inactivation at all of reductase phosphatases even at concentrations of 40 mM. It is known that monocarboxylic acids with more than two carbons show low chelating effect on divalent cations (28).

These results are summarized by the close correlation shown in Fig. 4, between I_{50} values and logarithm of the stability constants for all phosphatases and Mn^{2+} and Mg^{2+} salts. When log K increases, lower concentrations of the acid are necessary to produce inhibition. Correlations between I_{50} values and log K for Ca^{2+} or Sr^{2+} salts of the four carboxylic acids, produce lines situated to the left of the Mg^{2+} line in Fig. 4 (data not shown). As a consequence, the less stable Ca-protein complex would be produced if we admit Ca^{2+} (or Sr^{2+}) as possible candidates for the cation bound to protein. In support of this reasoning, it should be pointed out that Ca^{2+} was tested as an activator in a previous study (10) and almost no activation of reductase phosphatases was found.

Results of reductase phosphatase activities in the presence of different molar ratios of divalent cation to carboxylic acid are surprising; in most cases, a value of reductase phosphatase equal to the control was reached at ratios much lower than 1. The high K values of the assayed salts for Mg^{2+} and Mn^{2+} would suggest that concentrations of the free cation in solution would be in terms of micromolarities. However, these concentrations can counteract at least millimolar concentrations of polycarboxylic acids, producing full activity. This suggests that the affinity of Mn^{2+} or Mg^{2+} toward reductase phosphatase, using HMG-CoA reductase as substrate, is much higher than that of these ions to the carboxylic acids. Only when the concentration of acids is much higher than the concentration of the Mn^{2+} or Mg^{2+} supposedly present in the protein, is there inactivation reached as a result of the strong electric forces Me-ligand.

The lack of inhibition by EDTA reported in a previous study (29) may be explained by the structure of EDTA, which may restrict its access to the metal of the enzyme. This effect also observed with other protein phosphatases (13, 16, 32) has been explained previously by the fact that essential divalent cation may be buried in the active site of the enzyme.

Results of permanent inactivation of HMG-CoA reductase phosphatase by citrate in the absence of reductase, phosphorylase, and glycogen synthase, even after extensive dialysis of chelator, show that the inactivating effect is on the enzyme and not on its substrate.

The different behavior of divalent cations on the reactivation process may indicate the mechanism of action of the cation present in the assays. Mn^{2+} reactivates citrate-inactivated phosphatases Ia and IIa (high Mr forms) in a similar manner, independently of the substrate of the reaction. In contrast, Mg^{2+} produces reactivation in phosphatases Ia and IIa only when the substrate is HMG-CoA reductase. Using an Mr 250,000 form of active phosphorylase phosphatase, Brautigan et al. (15) reported that the activation by Mn^{2+} was not mimicked by Mg^{2+}. One explanation of these facts is that Mg^{2+} is not the cation bound to the phosphatase and only mimics the effect of Mn^{2+} depending on which substrate is present in the reaction. Alternatively, results with high Mr forms can also be interpreted to mean that each of the phosphatases Ia and IIa contains two distinct enzyme species. One can be specific for HMG-CoA reductase, and either Mn^{2+} or Mg^{2+} can satisfy its metal requirement. The other is specific for phosphorylase a and glycogen synthase D and only Mn^{2+} can satisfy its metal requirement. To our knowledge, there are no data on reactivation of either citrate-inactivated rat liver phosphorylase phosphatase or citrate-inactivated glycogen synthase phosphatase; as a consequence, the discussion of results of Table 3 is less conclusive.

The lack of reactivation of Mg^{2+} in the citrate-inactivated low Mr phosphatases Ib and IIb corresponds to similar results in polyphosphate-inactivated phosphorylase phosphatase previously described (14, 16, 32). On the other hand, the lack of reactivation of Mn^{2+} in inactivated phosphatases Ib and IIb with phosphorylase as substrate suggest to us that our preparations may be similar to those phosphatases cited by others (33–36) who have previously shown that active phosphorylase phosphatase preparations are not activated by Mn^{2+}. They may represent isozyme species from those phosphorylase phosphatases reported by others (13, 16, 31, 32) that can be reactivated by Mn^{2+}. This effect of Mn^{2+} on reductase phosphatases appears to be on the binding enzyme-substrate, as different behavior on the reactivation is found when the substrate is other than HMG-CoA reductase.

The low reactivation (52%) of Mn^{2+} on phosphatase Ib for glycogen synthase agrees with the results obtained by Khatra and Soderling (13) for ATP-inactivated gly-

cogen synthase phosphatase from rabbit muscle. The preparations of protein phosphatases of these authors probably contained both low Mr forms, similar to those that we described as Ib and IIb.

It has not escaped our notice that, depending upon the partitioning between cytosol and mitochondria, variations in carboxylate concentrations may regulate in vivo cholesterol biosynthesis.

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