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Inactivation of Rat Liver HMG-CoA Reductase Phosphatases by Nucleotides

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Incubation of four purified rat liver HMG-CoA reductase phosphatases, with ATP, ADP and AMP caused a concentration-dependent inactivation of enzyme activities. The nucleotides of guanine, cytosine and uracil produced similar effects to those by the nucleotides of adenine for the same number of phosphates present in the molecules. The greater the number of phosphate groups in nucleotides, the higher was the inhibition in reductase phosphatases observed. Preincubation of phosphatases with ATP and subsequent dilution did not diminish the inactivation effect, showing that nucleotides inhibit the enzyme prior to their binding to the substrate. A relationship was observed between those concentrations of nucleotides which produce 50% inactivation and the logarithm stability constant of Mg or Mn salts of nucleotides. ATP-inactivated enzymes were reactivated by Mn^{++} and to a lesser proportion by Mg^{++}, the conclusion being that HMG-CoA reductase phosphatases have the characteristics of metalloenzymes.

Cholesterol biosynthesis in the liver of a number of vertebrates is regulated principally through the microsomal enzyme 3-hydroxy 3-methylglutaryl coenzyme A reductase (NADPH) (E. C. 1.1.1.34) which catalyzes the rate-limiting reaction in this pathway (12, 13). Previous studies have shown that HMG-CoA reductase can be interconverted *in vitro* from an active to an inactive form. Microsomal (1, 2, 9, 17) and solubilized (8) reductase is inactivated *in vitro* in the presence of ATP-Mg and a reductase kinase with the covalent binding of phosphate in two sites or sets of sites to the reductase molecule (5). Inactivated homogeneous ^{32}P-labeled HMG-CoA reductase is reactivated by rat liver HMG-CoA reductase phosphatases with a concomitant release of ^{32}P (7).

The inactivating effect of inorganic phosphate and pyrophosphate on several protein phosphatases has been previously reported. BURCHELL and COHEN

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Abbreviations used:
HMG-CoA, 3-Hydroxy 3-menthyl glutaryl coenzyme A; Reductase, HMG-CoA reductase.

(4) proposed that rabbit muscle phosphorylase phosphatase is a metalloenzyme, when they observed the inactivation of the enzyme activity by phosphate and the reactivation by Mn^{++}. Analogous arguments were given by KANDELWAL and KAMANI (14) for rat liver phosphorylase phosphatase. In a previous work (10) we reported the inactivating effect of both phosphate and pyrophosphate on reductase phosphatases and the reversion of this effect by Mn^{++} and Mg^{++}.

In this study we show the inhibitory effect of the nucleotides mono-, di- and tri-phosphates on HMG-CoA reductase phosphatases from rat liver. We present evidence of the close relationship between capacity of chelation of these nucleotides (expressed either by the number of phosphates in their molecules, or by the stability constants of the divalent cation-nucleotide complexes) and their inhibitory properties on reductase phosphatases. Moreover, we can show the reversion of the inhibitory effect when reductase phosphatases are incubated with Mn^{++} or Mg^{++}. We propose from these data that HMG-CoA reductase phosphatases are metalloenzymes, with Mn probably being the metal bound to the enzyme.

Materials and Methods

Chemicals. Most of the reagents used in this study have been previously reported (7). Magnesium and manganese chloride salts were from Merck. All nucleotides used were from Sigma.

Buffered solutions. Buffer A is 100 mM sucrose, 40 mM potassium phosphate, 10 mM sodium pyrophosphate, 30 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, pH = 7.2. Buffer B contained 40 mM Bis-tris, 1 mM dithiothreitol, 300 mM sucrose, 0.05% (w/v) serum albumin pH = 6.5.

Purification of the enzymes. HMG-CoA reductase was solubilized from the 100,000 x*g* pellet of livers from rats fed 5% cholestyramine for 5 days. After solubilization by freeze-thawing and glycerol extraction (3) the enzyme was inactivated with 4 mM ATP and purified to homogeneity by affinity chromatography on Affi-Gel Blue and HMG-CoA Agarose (8). Under these conditions, the fraction of HMG-CoA reductase which remained in the active form was 11% (343 mU/mg protein), and therefore was a good substrate for the action of phosphatases. HMG-CoA reductase phosphatases were purified as described (7). Reductase phosphatases Ia and IIa are the purified reductase phosphatases of high molecular weight (480,000 and 310,000 daltons, 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 daltons) obtained after 95% ethanol treatment at room temperature from reductase phosphatases Ia and IIa. Specific activities were 330, 710, 11320 and 21480 μunits/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 (7, 8). In addition, the reductase phosphatases exhibited protein phosphatase activity towards homogeneous rabbit muscle phosphorylase a and homogeneous rat liver glycogen synthase D.

Enzymatic assays. HMG-CoA reductase activity was assayed as described (7). HMG-CoA reductase phosphatase activity was determined by measuring the increase in activity (reactivation) of a homogeneous HMG-CoA reductase which had been inactivated by phosphorylation. This was compared to a control without phosphatase. The assay was conducted as follows: Samples to be assayed (10 μl, 20 μunits) were mixed with homogeneous

HMG-CoA reductase (5 μl, 0.22 μg) and 10 μl buffer B with or without effector and incubated at 37° C. After 15 min the reaction was arrested by adding 350 μl buffer A. Aliquot portions of 50 μl of this mixture were incubated at 37° C for 30 min with 55 μl of a solution containing 22 μmol Tris/HCl, pH = 7.2, 0.5 U glucose 6 phosphate dehydrogenase, 5 μmol EDTA, 0.4 μmol dithiothreitol, 5 μmol glucose 6 phosphate, 0.3 μmol NADP, 0.05 mg serum albumin. This preincubation eliminated unlabeled HMG-CoA which accompanied purified HMG-CoA reductase. The reaction was started by adding 5 μl (11.2 μg) of [^{3}H] HMG-CoA (22,500 dpm/nmol) and further incubated for 15 min. The reaction was arrested with 40 μl 3 M HCl and incubated at 50° C for 10 min to convert mevalonate to mevalonolactone. Separation of [^{3}H] mevalonolactone and [^{3}H] hydroxymethylglutaric acid was performed by the «mixed phase» method proposed by PHILIPP and SHAPIRO (19). The amounts of reductase phosphatase assayed were such that less than 15% of the substrate was inactivated 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 see whether effectors do interact with the substrate HMG-CoA reductase. It was previously shown (8) that when an effector does not modify the activity of ^{32}P-labeled HMG-CoA reductase, its content in ^{32}P is equally unmodified.

Units. One unit (U) of HMG-CoA reductase was defined as the amount of enzyme which converts 1 μ mol HMG-CoA into mevalonate/min. One unit of HMG-CoA reductase phosphatase was defined as the enzyme which increase 1 U of HMG-CoA reductase/min. Specific activity was defined as U/mg protein.

Results

Inactivation of rat liver HMG-CoA reductase phosphatases by nucleotides mono-, di- and tri-phosphates. When reductase phosphatases Ia and Ib were incubated with HMG-CoA reductase and increasing amounts of ATP, ADP and AMP, a concentration-dependent inactivation was observed (fig. 1). Concentrations of 1 mM ATP or ADP produced full inactivation in both reductase phosphatases. However, at this concentration, AMP produced inactivations of only 30% in phosphatases Ia and Ib. cAMP did not

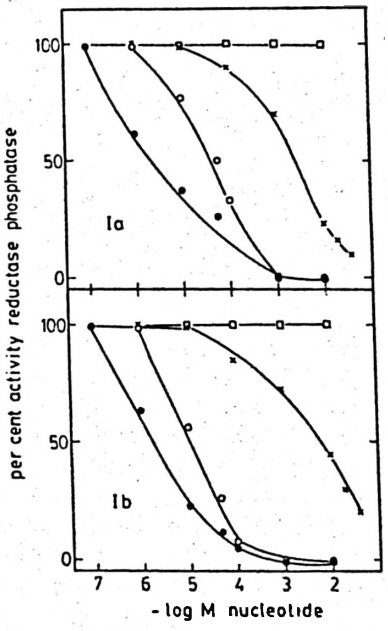

Fig. 1. *Inactivation of purified rat liver HMG-CoA reductase phosphatases by varying concentrations of nucleotides.*

Reductase phosphatases Ia and Ib (20 micro-units each) were incubated with homogeneous HMG-CoA reductase (0.22 μg) at 37° C with varying concentrations of ATP (●), ADP (○), AMP (x), and cAMP (□) and after 15 min incubation, aliquots were withdrawn, appropriately diluted with buffer A and assayed for HMG-CoA reductase. Reductase phosphatase activity without added nucleotides was taken as 100%. Upper panel: Reductase phosphatase Ia. Lower panel: Reductase phosphatase Ib.

Table I. *Effect of incubation with different nucleotides on the activities of purified rat liver HMG-CoA reductase phosphatases.*

Purified HMG-CoA reductase phosphatases Ia, IIa, Ib and IIb, 20 micro-units each, were incubated with homogenous HMG-CoA reductase (0.22 μg) and varying concentrations of different nucleotides in buffer B in a total volume of 25 μl. After incubation of 15 min at 37° C aliquot portions from each tube were withdrawn and diluted with 350 μl buffer A. HMG-CoA reductase activity was determined in diluted samples. Figures represent the concentrations of the effectors which produce 50 % inactivation on the reductase phosphatases. The logarithm stability constants for complexes of Mg and Mn of the nucleotides are given to the right.

Nucleotide	Reductase phosphatase (μM)				Log K (Mg)	Log K (Mn)
	Ia	IIa	Ib	IIb		
ATP	3	1	1	2	4.04	4.75
GTP	5	8	6	9	4.01	4.73
CTP	8	10	4	6	4.01	4.78
UTP	7	9	2	5	4.02	4.78
ADP	14	56	10	14	3.15	3.94
GDP	13	40	22	36	—	—
CDP	40	56	18	40	—	—
UDP	50	71	13	20	3.17	—
AMP	1 260	3 160	7 900	6 300	1.95	2.31
GMP	2 200	5 500	1 600	4 700	—	—
CMP	4 000	7 000	8 000	10 000	—	—
UMP	2 000	5 000	4 000	5 000	2.25	—

produce any significant effects at the concentration interval of 0.1—8 mM. Similar results were obtained when reductase phosphatases IIa and IIb were tested instead of phosphatases Ia and Ib.

The incubation of nucleotides mono-, di- and triphosphates of guanine, cytidine and uracil with the four reductase phosphatases Ia, IIa, Ib and IIb produced similar curves as adenine nucleotides, for the same number of phosphates present in molecules. The concentrations of the effectors which produce 50% inactivation of reductase phosphatases (I_{50}) are represented in table I. It is concluded that the more negative charged phosphate groups in the molecule of inactivators, the higher the inactivation of reductase phosphatase, being the base participant in the nucleotide less important in the inactivation effect.

We were interested in ascertaining whether nucleotides directly affected the phosphatases or the substrate, HMG-CoA reductase. To this end, each reductase phosphatase was incubated for 15 min at 37° C with ATP at concentrations ranging from 0.1 μM to 10 mM. After 15 min phosphatases a (high Mr forms) were diluted 50 fold while phosphatases b (low Mr forms) were diluted 200 fold. The reductase phosphatase activity using homogeneous HMG-CoA reductase as substrate was determined as described in Methods.

A continuous pattern of inactivation was attained for each phosphatase when concentrations of ATP were increased. Reductase phosphatase IIa was inactivated 50% by 0.01 mM ATP, in a preincubation period of 15 min. I_{50} values of reductase phosphatases Ib and IIb were 1 and 5 μM, respectively. Concentrations of about 1 mM produced complete inactivation of the four reductase phosphatases, as ocurred in the experiments in which ATP was present throughout. These results indicate that ATP interacts with the

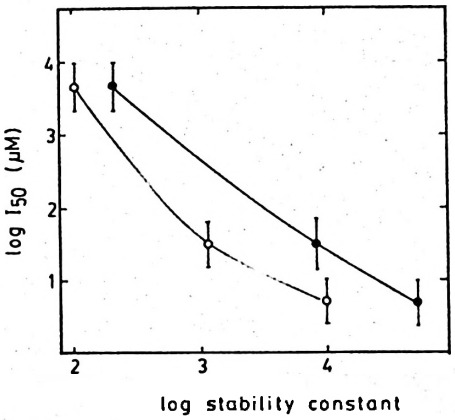

Fig. 2. *Correlation between the logarithm of stability constants of Mg (open symbols) and Mn (closed symbols) salts of nucleotides and the concentration of these which produce 50% inactivation (I_{50}) on the four reductase phosphatases.*
Values of the logarithm stability constants of the Mn-and Mg-nucleotide complexes are shown in table I.

enzyme and not with its substrate HMG-CoA reductase.

Relationship between I_{50} values and stability constants of Mg and Mn nucleotides. Nucleotides are chellators of divalent cations, due to the phosphate groups. The complex formed between the ligand and the metal is in equilibrium with the reactants, according to the following expression:

$$\text{Nucleotide}^{n-} + \text{Metal}^{++} \rightleftharpoons \rightleftharpoons \text{Me - Nucleotide}^{(n-2)-}$$

The stability constant is defined by the equation:

$$K = \frac{[\text{Me - Nucleotide}^{(n-2)-}]}{[\text{Nucleotide}^{n-}][\text{Me}^{++}]}$$

We considered it of interest to correlate the values of logarithm K for the different Mg and Mn salts of nucleotides (18) with

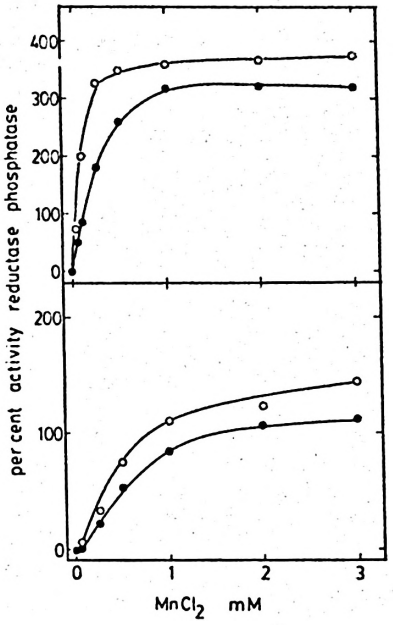

Fig. 3. *Reactivation of ATP-inactivated rat liver HMG-CoA reductase phosphatases by Mn^{++}.*
The inactivation of reductase phosphatases was carried out in the presence of 1 mM ATP. The diluted ATP-inactivated reductase phosphatases were assayed for activity in the presence of HMG-CoA reductase and different concentrations of Mn^{++}. Reductase phosphatases without added effectors were taken as 100%. Upper panel: High molecular weight reductase phosphatases Ia (●) and IIa (○). Lower panel: Low molecular weight reductase phosphatases Ib (●) and IIb (○).

the inactivation effect on reductase phosphatases expressed by the I_{50} value (Fig. 2). When log stability constants of complexes Me-Nucleotide increase in value, lower concentrations of nucleotide are necessary to inhibit 50% reductase phosphatases. Under these conditions, all reductase phosphatases behave in a similar manner. This correlation appears to hold, whether the divalent cation is Mn or Mg. From Fig 2 it can be deduced that in the hypothetical case that one of these two cations were to bind the protein, Mn appears to be more strongly bound than Mg, as higher concentrations of neucleo-

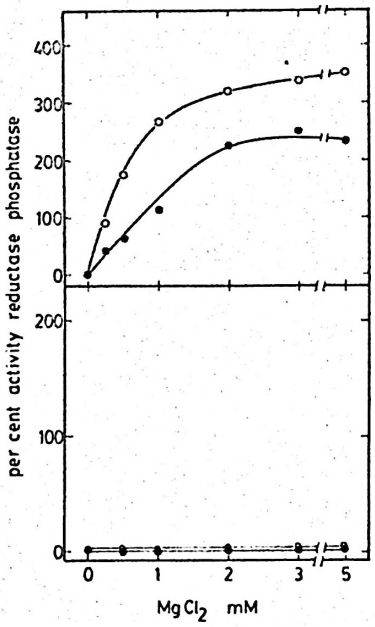

Fig. 4. *Reactivation of ATP-inactivated rat liver HMG-CoA reductase phosphatases by Mg^{++}*
Inactivation and reactivation methods were as described in fig. 3, but with 5 mM $MgCl_2$. Symbols are as in fig. 3.

tides are necessary to produce the same I_{50}, at a same stability constant value.

Reactivation of ATP-inactivated reductase phosphatases by Mn and Mg ions. As the inactivators tested in this study were, in fact, substances capable of sequestering the metal which is possibly present in the enzyme, we asked whether the inactivation produced by ATP and other nucleotides was reversibly counteracted by further incubation with divalent cations. For this purpose, aliquot portions of each reductase phosphatase were incubated for 15 min with 1 mM ATP for inactivation, dyalized or diluted 50 or 200 fold (phosphatases a or b respectively) and the HMG-CoA reductase phosphatase activity being measured in the presence of the divalent cations as described in Methods.

Fig 3 shows the effect of 5 mM Mn^{++} on reactivation of reductase phosphatases. High Mr phosphatases completely reactivated their activity at 0.1 mM Mn^{++}, while low Mr phosphatases Ib and IIb needed 1 mM Mn^{++} to reach original values (those before inactivation with ATP). Concentrations of about 1 mM Mn^{++} reactivated high Mr forms 300-350%.

The effect of Mg^{++} on reactivation of reductase phosphatases was also tested in an experiment similar to Mn^{++}. Fig 4 shows that concentrations 0.25 - 1 mM Mg fully reactivated high Mr forms and at 5 mM Mg^{++} the activity increased 200-350%; in an opposite way, low Mr forms do not reactivate at all even at concentrations as high as 5 mM Mg^{++}, showing that Mg^{++} produces no effect on these phosphatases.

Discussion

In a previous work (10), we suggested the metalloenzymatic character of reductase phosphatases on the basis of their activations by Mn and Mg ions, and inactivation by chelators of these Pi and PPi ions. Several authors had previously suggested similar conclusions for phosphorylase phosphatase of various origins (11, 15, 16).

This study reinforces the idea that reductase phosphatases from rat liver need a divalent cation in order for activity to take place. Nucleotides which chelate these ions inhibit the reductase phosphatase activity. There is a close correspondence between concentrations of ligand which produce 50% inactivation of reductase phosphatases and the log stability constant of Mn or Mg nucleotide complexes. When log K increases, lesser concentrations of the ligand are necessary to produce inhibition. Inactivation by nucleotides and reactivation by Mn^{++} and Mg^{++} are consistent with the idea that these reductase phosphatases are metal-

loenzymes. The stronger binding of Mn to the reductase phosphatase compared with Mg, deduced from results of Fig 2, and the higher reactivation of the ATP-inactivated phosphatases by Mn^{++} compared with Mg^{++} permits the assumption that Mn is the metal present in reductase phosphatases.

The inhibitory properties of reductase phosphatases of the substrates of HMG-CoA reductase, NADPH, HMG-CoA and CoA previously reported by us (6) which produced analogous curves to those of nucleotides di- and triphosphates, indicate that the actual inhibitor probably is the pyrophosphate group present in all these molecules. The comparison between inhibition produced by inorganic pyrophosphate on reductase phosphatases (10) and those inhibitions produced by organic molecules which contain it, permits the assumption that all the responsability of inactivation is due to the pyrophosphate itself, being the contribution to the inactivation of the rest of the molecule much less important.

The inactivating effect of nucleotides may conceivably exert a regulatory influence on reductase dephosphorylation and activation *in vivo*. However, the precise influence of the change of neucleotide concentrations on reductase phosphatases *in vivo* is difficult to ascertain, as the phosphate and pyrophosphate groups of nucleotides chellate Mg^{++} and Mn^{++} ions, present in the cell, and thus the inactivating capacity of nucleotides is disminished; on the other hand, the differential effect of changes in ATP and other nucleotides, on every reductase phosphatase should be observed after the separation of the four phosphatases. The influence of several hormones on reductase phosphatases in experiments *in vivo* is subject of current investigation.

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Resumen

La incubación de las cuatro HMG-CoA reductasa fosfatasas purificadas a partir de hígado de rata con ATP, ADP, AMP, produce inactivación de las actividades enzimáticas dependiente de la concentración del nucleótido. Los nucleótidos de guanina, citosina y uracilo con un mismo número de fosfatos presentes en la molécula, producen efectos análogos a los nucleótidos de adenina. Cuanto mayor es el número de fosfatos, mayor la inhibición en las reductasa fosfatasas. La preincubación de las fosfatasas con ATP, y dilución posterior no disminuye el efecto inactivante, concluyéndose que los nucleótidos inhiben las fosfatasas antes de su unión al substrato. Se observa que existe una correlación entre aquellas concentraciones de nucleótidos que producen un 50% de inactivación y el logaritmo de la constante de estabilidad de los complejos Mn- o Mg-nucleótido. Las HMG-CoA reductasa fosfatasas inactivadas por ATP son reactivadas por Mn^{++} y en menor proporción por Mg^{++}, concluyéndose que son metaloenzimas.

References

1. Beg, Z. H., Stonik, J. A. and Brewer, H. B.: *J. Biol. Chem.*, 255, 8541-8545, 1980.
2. Bové, J. and Hegardt, F. G.: *FEBS Lett.*, 90, 198-202, 1978.
3. Brown, M. S., Dana, S. E., Dietschy, J. M. and Siperstein, M. D.: *J. Biol. Chem.*, 248, 4731-4738, 1973.
4. Burchell, A. and Cohen, P.: *Biochem. Soc. Transac.*, 6, 220-222, 1978.
5. Font, E., Sitges, M. and Hegardt, F. G.: *Biochem. Biophys. Res. Commun.*, 105, 705-710, 1982.
6. Gil, G. and Hegardt, F. G.: *Arch. Biochem. Biophys.*, 214, 192-198, 1982.
7. Gil, G., Sitges, M. and Hegardt, F. G.: *Biochim. Biophys. Acta*, 663, 211-221, 1981.
8. Gil, G., Sitges, M. and Hegardt, F. G.: *Arch. Biochem. Biophys.*, 210, 224-229, 1981.
9. Gil, G., Sitges, M., Bové, J. and Hegardt, F. G.: *FEBS Lett.*, 110, 195-199, 1980.
10. Gil, G., Calvet, V. E., Ferrer, A. and Hegardt, F. G.: *Hoppe Seyler's Z. Physiol. Chem.*, 363, 1217-1224, 1982.

11. HSIAO, K. J., SANDBERG, A. R. and LI, H. C.: *J. Biol. Chem.*, 253, 6901-6907, 1978.
12. HUNTER, C. F. and RODWELL, V. W.: *J. Lipid Res.*, 21, 399-405, 1980.
13. INGEBRITSEN, T. S. and GIBSON, D. M. in «Molecular Aspects of Cellular Regulation» (Cohen, P. ed.) Vol I, Elsevier/North Holland, Amsterdam, 1980. pp 63-93.
14. KHANDELWAL, R. L. and KAMANI, S. A. S.: *Biochim. Biophys. Acta*, 613, 95-105, 1980.
15. KHATRA, B. S. and SODERLING, T. R.: *Biochem. Biophys. Res. Commun.*, 85, 647-654, 1978.
16. MACKENZIE, C. W., BULBULIAN, G. and BISHOP, J. S.: *Biochim. Biophys. Acta*, 614, 413-424, 1980.
17. NORDSTROM, J. L., RODWELL, V. W. and MITSCHELEN, J. J.: *J. Biol. Chem.*, 252, 8924-8934, 1977.
18. O'SULLIVAN, W. J. In «Data for Biochemical Research» (Dawson, R. M. C., Elliot, D. C., Elliot, W. H. and Jones, K. M., eds.) 2nd ed. Oxford University Press. London, 1969. pp 423-434.
19. PHILIPP, B. W. and SHAPIRO, D. J.: *J. Lipid Res.*, 20, 588-593, 1979.