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Antibody Preference for the Catalytically Active Form of β-Hydroxy-β-Methylglutaryl Coenzyme A Reductase

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Abstract

The catalytically inactivating subset within rabbit serum polyclonal antibody to the solubilized, purified 55,000 to 60,000 dalton active fragment of rat liver microsomal β-hydroxy-β-methylglutaryl coenzyme A reductase immunoinactivates this enzyme with little or no diminution of effect by enzyme catalytically inactivated by incubation of microsomes with ATP,Mg^{++}. Reactivation of inactive enzyme with ethanol-treated rat liver phosphatase restores antibody affinity showing that the catalytically inactivating subset of antibody exhibits marked or complete affinity for the active enzyme over the ATP,Mg^{++}-inactivated form. This means that immunoinactivation using this antibody is not a valid way of measuring changes in the specific activity of the enzyme via phosphorylation–dephosphorylation. Preference for the active enzyme has not been obvious because when different amounts of enzyme activity are used in immunotitrations of samples of low activity, apparent differences in specific activity are observed when none actually exist. If precautions are not taken, results are obtained supporting phosphorylation by using an antibody that is not capable of distinguishing it.

Key Words: Immunoinactivation; immunotitrations; antibody preference; phosphorylation of enzymes; HMG-CoA reductase, regulation of; cholesterol synthesis, regulation of.

Introduction

Recently, assays utilizing the immunoinactivation of enzymes have come to be frequently employed as a means of determining enzyme regulatory mechanisms. This approach has been used in a number of laboratories to study the

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regulation of hepatic cholesterol biosynthesis by measuring the immunoinactivation of HMG-CoA[3] reductase as a function of changes in the animals' nutritional or hormonal state (Hardgrave *et al.*, 1979; Edwards *et al.*, 1979; Kleinsek *et al.*, 1980; Arebalo *et al.*, 1980; Edwards *et al.*, 1980; Jenke *et al.*, 1981; Arebalo *et al.*, 1982a; Dugan *et al.*, 1982). It is unfortunate that the results obtained in different laboratories by immunoassay procedures have led to conflicting conclusions about the mechanism of enzyme regulation. For example, immunoassays in one laboratory (Arebalo *et al.*, 1982a) indicated that the inhibition of hepatic HMG-CoA reductase activity induced by short-term cholesterol feeding to rats results from an increase in the rate of inactivation of preexisting enzyme, whereas, in two other laboratories (Jenke *et al.*, 1981; Dugan *et al.*, 1982), a conclusion was reached that the inhibition did not result from inactivation because no change was detected in the proportion of active and inactive enzyme. Different conclusions have also been reached when immunoassays were used to determine the mechanism of inhibition of rat liver HMG-CoA reductase activity by mevalonolactone (Arebalo *et al.*, 1982a; Dugan *et al.*, 1982), as well as its stimulation by cholestyramine feeding (Edwards *et al.*, 1979; Kleinsek *et al.*, 1980) and during the diurnal cycle (Kleinsek *et al.*, 1980; Edwards *et al.*, 1980).

One preliminary report (Arebalo *et al.*, 1982b) has suggested a reason for conflicting conclusions by claiming a difference in antibody preference for forms of the enzyme as a function of time into the immunization schedule. The results obtained indicated that early collections of antisera, one to two months, preferentially bind to the active form of HMG-CoA reductase, whereas later antisera are nonpreferential. This raises the possibility that different interpretations stemmed from different times of collection of the antibody.

Two assumptions have been made in immunoinactivation studies previously conducted. One is that the degree of immunoinactivation is independent of the quantity of enzyme activity reacted with antibody. The other is that the subset of polyclonal antibodies inactivating HMG-CoA reductase has equal affinity for active and inactive forms of the enzyme. If the former supposition is untrue, precautions are necessary to obtain results from which valid interpretations can be made about relative specific activities being proportional to degrees of immunoinactivation. If the latter presumption concerning equal affinity is untrue, then specific activity is not a function of degree of immunoinactivation when the preference for one form is complete. If preference is only partial, a much more complex situation exists than with no preference.

In the present study these two suppositions have been tested for the

[3]The following abbreviations were used: HMG, β-hydroxy-β-methylglutaryl; IgG, immunoglobulin G.

inactivating subset of a polyclonal antibody to rat liver HMG-CoA reductase. The findings lead to a comment on the validity of the assumptions in immunoinactivation studies and an explanation for the disparity of previous results.

Materials and Methods

Reagents

The chemicals used were obtained from the following sources: [2-^{14}C]β-hydroxy-β-methylglutaric acid from New England Nuclear; β-hydroxy-β-methylglutaric acid and NADP from Sigma; CoA and ATP from Pharmacia P-L Biochemicals; Freund's complete and incomplete adjuvant from Difco; and insulin (Iletin) from Eli Lilly. [2-^{14}C]HMG-CoA (800 dpm/nmol) was synthesized from the anhydride of β-hydroxy-β-methylglutaric acid which was prepared with dicyclohexylcarbodiimide (Goldfarb and Pitot, 1971).

Treatment of Animals, Preparation of Antigen and Antibody

The treatment of rats fed an *ad libitum* diet containing cholestyramine and subjected to a controlled light–dark cycle, the preparation of liver microsomes, solubilization and purification of HMG-CoA reductase, and preparation of antibody from the serum of a rabbit challenged with homogeneous HMG-CoA reductase have been described previously (Dugan *et al.*, 1982). The antibody used in this study is the same preparation.

Inactivation of Microsomal HMG-CoA Reductase with ATP,Mg^{++}

Microsomes were incubated 2 h at 37°C with and without addition of neutralized ATP and $MgCl_2$, each made 7 mM in an incubation mixture containing potassium phosphate, 100 mM, pH 7.0, and dithiothreitol, 2 mM. Triple dialysis was then carried out in the phosphate buffer with dithiothreitol and EDTA, 12.5 mM, in place of ATP,Mg^{++}. In the determinations so indicated, 25 mM potassium fluoride was also present in both buffers. Microsomes were pelleted by centrifugation and stored at −70°C until resuspension just before assays were performed.

Preparation of Phosphatase

Phosphatase was prepared from rat liver similar to the procedure described for the ethanol-treated preparation from rabbit liver (Brandt *et al.*, 1975). The protein in the supernatant solution of rat liver homogenate after

28,000 × g centrifugation for 60 min was precipitated with 70% saturation ammonium sulfate. The pellet was dissolved in and dialyzed against 50 mM imidazole buffer, pH 7.45. The solution was mixed with five volumes of ethanol and centrifuged at 5,000 × g for 5 min. The pellet was homogenized with imidazole buffer and the mixture was centrifuged at 16,000 × g for 15 min. The supernatant solution was dialyzed against imidazole buffer and stored frozen for use in phosphatase studies.

Reactivation of ATP,Mg^{++}-Inactivated HMG-CoA Reductase

Microsomal HMG-CoA reductase (~0.3 mg) was incubated with an equal amount of protein of partially purified, ethanol-treated rat liver phosphatase for 1 h at 37°C and for an additional hour in the presence or absence of antibody. No fluoride was present either in the preparation of microsomal enzyme or its incubation with phosphatase.

Inactivation of Microsomal HMG-CoA Reductase with Polyclonal Antibody

Microsomal HMG-CoA reductase was incubated with 2.5 μg of purified rabbit serum immunoglobulin G for 1 h at 37°C.

Assay of HMG-CoA Reductase

The radioassay was carried out as previously described (Kleinsek *et al.*, 1981) except that 0.4 M potassium chloride was in the assay mixture.

Assay of Mixtures of Active and ATP,Mg^{++}-Inactivated Microsomal HMG-CoA Reductase

Microsomal protein containing HMG-CoA reductase activity was mixed with an equal portion of ATP,Mg^{++}-incubated protein which was 100% enzymatically inactive if fluoride was present, and 85% inactive if fluoride was absent from the buffers. Prior to mixing, ATP and Mg^{++} were removed by triple dialysis in buffer containing 12.5 mM EDTA.

Results

Immunoinactivations with Fixed Antigen and with Fixed Antibody

The immunoinactivation of HMG-CoA reductase as a function of the quantity of antibody reacted is shown in Fig. 1. The amount of inactivation is directly proportional to the amount of antibody added at high ratios of the enzyme–antigen to the antibody. Only in the region where the amount of

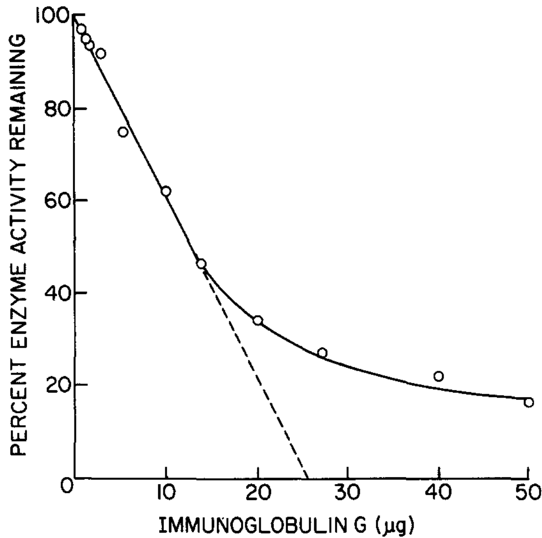

Fig. 1. Immunoinactivation of HMG-CoA reductase as a function of the amount of antibody added. Samples (0.25 ml) containing 0.113 mg of rat liver microsomal protein and HMG-CoA reductase activity of 400 pmol mevalonic acid formed per minute were incubated with the amount of IgG-containing antiHMG-CoA reductase shown on the figure. Assays were then carried out for the remaining HMG-CoA reductase activity.

inactivation increases linearly with the amount of antibody is the amount of inactivation independent of the amount of enzyme activity.

The immunoinactivation of HMG-CoA reductase as a function of quantity of enzyme activity is shown in Fig. 2. In samples from cholestyramine-fed animals where the quantity of HMG-CoA reductase activity in rat liver is relatively high, 2.27 nmol mevalonic acid formed/min × mg protein, the amount of immunoinactivation produced through the range of 0.2 to 0.5 mg protein by 5 μg of antibody-containing immunoglobulin G is not dependent on the quantity of enzyme activity present. On the other hand, HMG-CoA reductase activity in the livers of diabetic rats is comparatively low, 0.16 nmol mevalonic acid formed/min × mg protein, even after 1 h of insulin stimulation. In this case the interaction of a similar range of protein with 1.5 μg of the immunoglobin G does not provide an antigen/antibody ratio large enough so that inactivation is independent of the quantity of enzyme activity present.

Measurement of Preferential Affinity of the Antibody

In order to determine if the HMG-CoA reductase-inactivating subset of polyclonal antibody had the same or different affinity for catalytically active

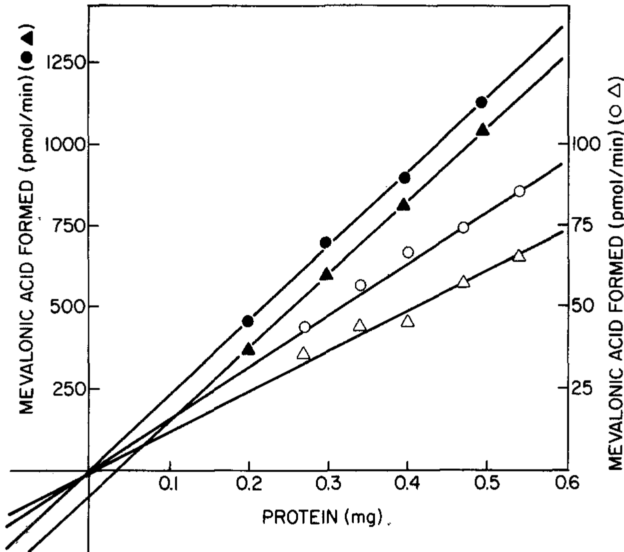

Fig. 2. Immunoinactivation of HMG-CoA reductase as a function of amount of enzyme added. Samples (0.25 ml) containing protein from rat liver were incubated 1 h at 37C with and without IgG-containing HMG-CoA reductase antibody and then assayed for HMG-CoA reductase activity. Lines were positioned according to linear regression analysis. (●) Microsomes from cholestyramine-fed rats; (▲) as before with 5 μg IgG; (O) protein solubilized from microsomes of diabetic rats treated by tail vein injection of insulin (3 units/100 g body weight) 1 h before being killed; (Δ) as before with 1.5 μg IgG.

Table I. Effect of Inactive HMG-CoA Reductase on the Immunoinactivation of the Active Enzyme[a]

Treatment	HMG-CoA reductase specific activity ± S.E. (pmol mevalonate formed $min^{-1} \times$ mg $protein^{-1}$)	HMG-CoA reductase specific immunoinactivation ± S.E. (pmol mevalonate lost $min^{-1} \times \mu g$ IgG^{-1})
Liver microsomes from cholestyramine-fed rats incubated without ATP,Mg^{++}	737 ± 5.24	32.3 ± 1.6
Above microsomes incubated with ATP,Mg^{++} followed by triple dialysis in EDTA-containing buffer	0	Not measurable
Mixtures of equal protein from each of above	358 ± 3.70	31.1 ± 2.4

[a] Means and standard errors are for 12 determinations. Samples containing 300 μg of HMG-CoA reductase-active microsomal protein with or without an equal portion of enzyme-inactive microsomal protein were assayed after a preincubation with or without 2.5 μg of IgG. Fluoride, 25 mM, was present in all incubations.

reductase and the enzyme inactivated with ATP,Mg^{++}, mixtures of the two and active enzyme alone were assayed for catalytic activity and immunoinactivation. The term "specific immunoinactivation" is used to represent the amount of inactivation per microgram of immunoglobulin G. The values in Table I can be compared. Addition of equal protein of ATP,Mg^{++}-inactivated enzyme to active enzyme had no effect on the total activity mixed, thereby decreasing the specific activity of active enzyme twofold. However, addition of ATP,Mg^{++}-inactivated enzyme reduced the degree of immunoinactivation only 7.4%. With equal affinity of antibody for the active and inactive forms the degree of immunoinactivation would be reduced 50%.

Recovery of Antibody Affinity with Recovery of Catalytic Activity

Incubation of rat liver microsomes with ATP,Mg^{++} in the absence of fluoride produced more than a fivefold decrease in the catalytic activity of HMG-CoA reductase, and a large apparent decrease in immunoinactivation (Table II). However, increasing the ratio of antigen to antibody reversed this decrease. An equal mixture of the active and partially inactivated HMG-CoA reductase-containing microsomes had an intermediate catalytic activity, but an immunoinactivation equivalent to undiluted active HMG-CoA reductase-containing microsomes, indicating no effect on immunoinactivation by ATP,Mg^{++}-produced inactive HMG-CoA reductase. Phosphatase reactivation mostly restored catalytic activity and fully restored the apparent loss in immunoinactivation exhibited by 0.3 mg of microsomal protein that had been ATP,Mg^{++}-incubated.

Table II. Effect of Recovery of Catalytic Activity on the Immunoinactivation of HMG-CoA Reductase[a]

Treatment	HMG-CoA reductase specific activity (pmol mevalonate formed $min^{-1} \times$ mg $protein^{-1}$)	HMG-CoA reductase specific immunoinactivation (pmol mevalonate lost $min^{-1} \times \mu g\ IgG^{-1}$)
0.3 mg of liver microsomes from cholestyramine-fed rats, not preincubated	763	31.1
1.5 mg, not preincubated	540	31.8
0.3 mg of above preincubated with ATP,Mg^{++}	140	7.2
1.5 mg preincubated with ATP,Mg^{++}	113	26.0
0.3 mg not preincubated, mixed with 0.3 mg preincubated with ATP,Mg^{++}	436	32.9
0.3 mg preincubated with ATP, Mg^{++}, followed by incubation with the catalytic subunit of rat liver phosphatase	597	33.6

[a]Fluoride was absent from all buffers.

Discussion

The high ratios of antigen to antibody required for degree of inactivation to be independent of quantity of antigen, e.g., enzyme activity (Figs. 1 and 2), cannot be obtained from nutritional or hormonal states where the enzyme is greatly depressed. If the depressed state is compared to itself or another state where more enzyme activity is reacted with fixed amount of antibody, a result indicating a higher specific activity is obtained when more activity is reacted even if the amount of protein is the same in each. This apparent difference, when none exists, is not obtained if equal amounts of enzyme activity from each state are reacted with a fixed amount of antibody. The use of equal amounts of enzyme activity from each state becomes a requirement, therefore, when degree of inactivation is dependent on amount of enzyme activity present.

An additional requirement for relative degrees of immunoinactivation to represent relative specific activities of an enzyme is that the antibody must have equal affinity for active and inactive forms of the enzyme. Clearly this is not true for the enzyme-inactivating subset within polyclonal antibody to a solubilized, purified, enzymatically active fragment of microsomal rat liver HMG-CoA reductase. The antibody shows almost complete preference for the active form over the form of the enzyme inactivated by incubation with ATP,Mg^{++}. Furthermore, the lost affinity for the antibody by inactivated HMG-CoA reductase is restored during reactivation of HMG-CoA reductase by incubation with phosphatase. This shows the reversibility of the effect.

The assumption that the structural change causing enzyme inactivation during ATP,Mg^{++} incubation will not affect the affinity for antibody does not hold. According to the reports from several laboratories (Beg *et al.*, 1978; Keith *et al.*, 1979; Gil *et al.*, 1980; Beg *et al.*, 1980; Gil *et al.*, 1981; Beg and Brewer, 1982), this structural change is phosphorylation of HMG-CoA reductase. Since *in vitro*-phosphorylated HMG-CoA reductase loses affinity for catalytically inactivating antibody, the question arises if *in vivo*-phosphorylated reductase behaves the same. However, our experiments to effect *in vivo* phosphorylation with concomitant inactivation have not been successful (Dugan *et al.*, 1982).

The laboratories (Beg *et al.*, 1978; Keith *et al.*, 1979; Gil *et al.*, 1980; Beg *et al.*, 1980; Gil *et al.*, 1981; Beg and Brewer, 1982) that have reported *in vitro* phosphorylation have proposed phosphorylative inactivation as a short-term regulatory mechanism for HMG-CoA reductase and cholesterol biosynthesis. One laboratory has also reported (Beg *et al.*, 1980; Beg and Brewer, 1982) *in vivo* phosphorylation under hormonal control in further support of this proposal. On the other hand, other evidence (Ness *et al.*, 1980, 1982) indicates that the phosphorylation occurs on the product, mevalonate. This would result in the apparent inhibition attributed to reductase kinase, and it would cast

doubt on that enzyme's existence. In line with this proposal are reports (Jenke *et al.,* 1981; Dugan *et al.,* 1982) that nutritional and hormonal regulation are not by phosphorylative mechanisms. The case against phosphorylative regulation and in favor of regulation by changes in enzyme quantity has been reviewed by Ness (1983). Precautions were taken in these experiments to avoid apparent inhibition due to mevalonate kinase-catalyzed conversion of mevalonic acid to mevalonic phosphate which could produce the inhibition observed here. The ATP,MG^{++}-incubated microsomes were dialyzed three times in buffer containing 12.5 mM EDTA, then sedimented by centrifugation and resuspended and assayed in the presence of EDTA.

The results in Table II indicating a decrease in specific activity on phosphorylation of HMG-CoA reductase is shown to be reversed by increasing the antigen/antibody ratio. This result points out again that comparisons at low antigen/antibody ratios indicate different specific activities when there is actually no difference. Because of this, immuno-studies of our own (Dugan *et al.,* 1982) and perhaps others have indicated a different specific activity for HMG-CoA reductase after incubation of microsomes with ATP,Mg^{++}. These results should be reevaluated as evidence neither for nor against a change in specific activity.

The question of a phosphorylative mechanism aside, the principal conclusion from this study is antibody preference and its impact on the validity of immunotitrations. A small change in protein structure such as the addition of a phosphate can eliminate the binding affinity for a polyclonal subset of enzyme-inactivating antibodies. Presumably a monoclonal member of this subset would also not be bound by the inactivated HMG-CoA reductase. The probability that antibody preference is a property of all antibody preparations to the active form of HMG-CoA reductase, and the probability that antibodies to other enzymes show preference for one form should be of interest.

Acknowledgments

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