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REVERSIBLE PHOSPHORYLATION OF 3-HYDROXY-3-METHYLGLUTARYL CoA REDUCTASE IN MORRIS HEPATOMAS

Ronald G. Gregg and Peter A. Wilce

Department of Biochemistry, University of Queensland, St. Lucia, Queensland, Australia

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Summary: The reversible phosphorylation of microsomal 3-hydroxy-3-methylglutaryl CoA reductase in host liver and hepatoma 5123C has been investigated. The percentage of the total enzyme activity *in vivo* was similar in the normal liver, host liver and hepatoma 5123C. The inclusion of 30 mM EDTA and 10 mM mevalonic acid in assays of 3-hydroxy-3-methyl-glutaryl CoA reductase inactivation *in vitro* eliminated artifacts generated by the presence of mevalonate kinase. Inactivation of 3-hydroxy-3-methyl glutaryl CoA reductase from normal liver, host liver and hepatoma occurred at a similar rate with similar half-times. We conclude that phosphorylation/dephosphorylation of 3-hydroxy-3-methylglutaryl CoA reductase occurs in hepatomas and that the lack of dietary cholesterol feedback inhibition in the hepatomas is not a result of a defect in this particular aspect of the reversible phosphorylation system.

Introduction: The proposal that 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase, EC 1.1.1.34) undergoes short-term regulation by a cyclic phosphorylation/dephosphorylation mechanism has been the subject of recent reviews (1, 2, 3, 4). There is considerable evidence for inactivation of the enzyme by phosphorylation after animals are treated with cholesterol and mevalonic acid (3, 5, 6, 7). Ness *et al.* (8) point out that the inactivation by ATP(Mg) and a cytosolic protein (reductase kinase) may be an artifact generated by mevalonate kinase activity converting the mevalonate produced in the HMG-CoA reductase assay to a compound that is excluded from the extraction procedure. Although reductase kinase and mevalonate kinase activity have now been separated (9, 10), the work of Ness *et al.* (8) has emphasised that care must be taken in selecting the conditions for the assay of enzyme inactivation *in vitro*. Several protocols have been reported. Harwood and Rodwell (9) described a two-stage assay. During the first stage HMG-CoA reductase was inactivated and in the second

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stage HMG-CoA reductase activity was measured. NaF (76 mM), EDTA (76 mM) and mevalonic acid (114 mM) was included during the assay of HMG-CoA reductase activity to prevent activation by phosphatases and loss of labelled mevalonate by mevalonate kinase activity. Beg and Stonik (10) included a 30 fold excess of EDTA or 10 mM mevalonic acid in the assay.

The inhibitory effect of dietary cholesterol (11) or mevalonic acid treatment (12) is absent or reduced in Morris hepatomas. The defect, however, has yet to be described. One possibility is an altered state of phosphorylation of the enzyme *in vivo* or a defective phosphorylation/dephosphorylation system. We have investigated these possibilities in the experiments reported in this paper. We describe assay conditions of HMG-CoA reductase inactivation which exclude the artifactual influence of mevalonate kinase and show that reductase kinase is present in the host liver and the hepatoma 5123C. Further, the percentage of active enzyme in the hepatoma is similar to that of the normal liver and the phosphatase responsible for activation of HMG-CoA reductase is present in hepatoma. We conclude that the defect in dietary feedback regulation cannot be traced to these parameters.

Methods and Materials:

Determination of active and total HMG-CoA reductase

Animals were housed and killed, and a liver microsomal fraction prepared as previously described (13). To estimate total enzyme activity, the tissue was divided in two, microsomes were isolated from one half and assayed in buffer 1 (0.1 M-sucrose, 50 mM-KCl, 30 mM-Na_2EDTA, 40 mM-potassium phosphate, 5 mM-DTT, pH 7.6), while the proportion of enzyme active *in vivo* was estimated by preparing microsomes from the second half of the tissue in buffer 2 (buffer 1 with the addition of 50 mM NaF).

ATP(Mg) inactivation of HMG-CoA reductase

Washed microsomes were prepared in buffer 1 but resuspended in buffer 3 (buffer 1 containing 1 mM EDTA). All incubations were at 37°C. Microsomal membranes (1.2 ml) were incubated for 20 minutes to fully activate the enzyme, then 0.8 ml of buffer 3 containing ATP, $MgCl_2$ and NaF (5, 10 and 125 mM respectively) was added. At various times, 0.1 ml aliquots were removed and added to assay-tubes kept on ice and containing 0.04 ml of buffer 4 (buffer 1 containing 112.5 mM EDTA and 50 mM NaF). Assays were conducted in the presence of glucose 6-phosphate, $NADP^+$ and glucose 6-phosphate dehydrogenase (30 mM, 3 mM and 0.2 I.U. respectively). All assays were processed as described by Gregg and Wilce (14).

Results: The phosphorylation state of HMG-CoA reductase *in vivo* was investigated using homogenisation and assay buffers with and without 50 mM NaF. The data is shown in Table 1. The percentage of the total enzyme active *in vivo* was 15-20%. The percentage of enzyme active is not significantly different in the host liver or hepatoma 5123C when compared to normal liver.

To determine the reductase kinase inactivation of HMG-CoA reductase, assay conditions must satisfy two criteria. Both, the inactivation of HMG-CoA reductase in stage 2 of the assay and the loss of radio-labelled mevalonate by conversion to mevalonate phosphate by mevalonate kinase must be prevented.

To examine the first criteria, the addition of ATP(Mg) to the standard assay (including 30 mM EDTA) at various times was used. The data in Table 2 shows the effect of preincubation or assay in the presence of ATP, $MgCl_2$ and NaF (2, 4, 50 mM respectively). It can be seen that the presence of 30 mM EDTA completely inhibits the ATP(Mg) inactivation of the enzyme that occurs in the presence of 1 mM EDTA. To insure that labelled mevalonate is not lost from the assay 10 mM unlabelled mevalonic acid was included. Fig.1 shows that inactivation of HMG-CoA reductase from normal liver, in the presence and absence of 10 mM mevalonic acid is identical. These data establish that, using the experimental protocol above, the inactivation of HMG-CoA reductase is not an artifact of the assay conditions.

Table 1. Percentage of active HMG-CoA reductase in normal and host liver and hepatoma 5123C.

Tissue	% Active	No. Experiments
Normal liver	15.0 ± 2.7	5
Host liver	14.9 ± 3.2	5
Hepatoma 5123C	21.4 ± 4.3	5

Washed microsomal membranes were isolated and enzyme assayed in triplicate, either in the presence (active fraction) or absence (total activity) of 50 mM NaF. For each experiment microsomal membranes were isolated from the pooled tissue from two animals. Data is the mean ± SEM of the number of experiments indicated.

Table 2. Effect of ATP(Mg) on the activity of HMG-CoA reductase.

Assay Conditions	Activity (% Control)	
Control (no ATP(Mg), 30 mM EDTA)	100	(4)
Control (no ATP(Mg), 1 mM EDTA)	100	(4)
Preincubated (ATP(Mg), 30 mM EDTA, 50 mM NaF)	96.7 ± 4.7	(4)
Preincubated (ATP(Mg), 1 mM EDTA, 50 mM NaF)	9.3 ± 0.6	(3)
ATP(Mg) added after preincubation (30 mM EDTA, 50 mM NaF)	97.8 ± 5.3	(4)

Washed microsomal membranes were prepared in buffer 1, resuspended and preincubated in the presence of either 1 mM or 30 mM EDTA as indicated. All assays were conducted in triplicate in the presence of 30 mM EDTA. Preincubation was for 20 minutes and incubation for 5 minutes at 37°C. 2.0 mM ATP, 4.0 mM $MgCl_2$ and 50 mM NaF were added as indicated.

Fig. 2 shows the time course of inactivation of microsomal HMG-CoA reductase from Morris hepatoma 5123C and host liver. The specific activity of normal liver (Fig. 1), host liver and hepatoma 5123C at zero time was 0.29, 0.15 and 1.24 nmol mevalonate formed per minute per mg of microsomal protein respectively.

The half times of inactivation (assuming an exponential decrease) calculated after 10 minutes inactivation are 3.8, 4.3 and 3.9 minutes for

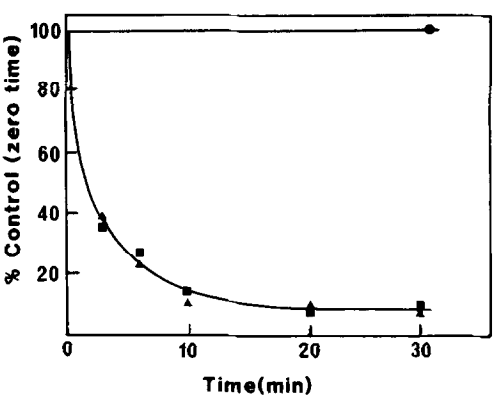

Figure 1. Time course of inactivation of microsomal HMG CoA reductase from normal liver in the presence and absence of 10 mM mevalonic acid. Washed microsomal membranes and enzyme assays were exactly as described in Methods. Control data is for assays without ATP(Mg) (●). Inactivation by ATP(Mg) is either in the presence (■) or absence (▲) of 10 mM mevalonic acid. Each point is the mean of duplicate assays.

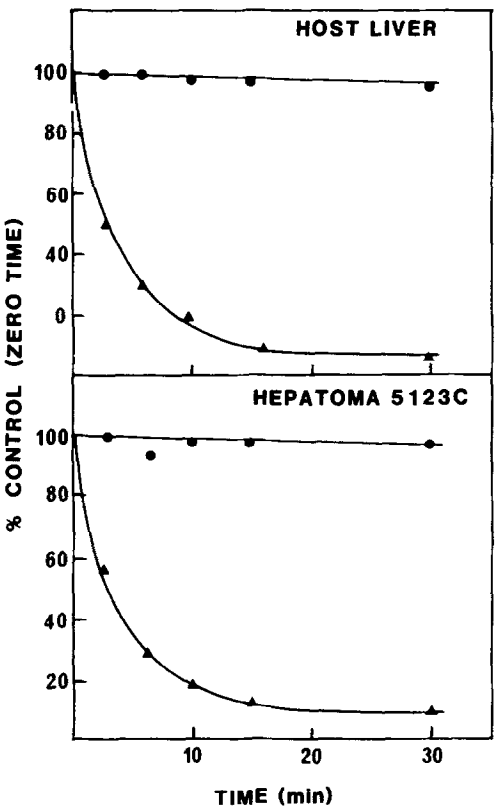

Figure 2. Inactivation of microsomal HMG CoA reductase by ATP(Mg) of host liver and hepatoma 5123C. Washed microsomal membranes were prepared and inactivation achieved exactly as described in Methods. Points are the mean of duplicate assays at the indicated time. All data are compared to the zero time control value. (●) Control (no ATP(Mg)); (▲) ATP(Mg) inactivation.

normal liver, host liver and hepatoma 5123C, respectively. Thus these results show that HMG-CoA reductase from host liver and hepatoma 5123C can be inactivated by ATP(Mg) and reductase kinase and that the proportion of active HMG-CoA reductase in the hepatoma is similar to normal liver.

Discussion: The mechanism responsible for the loss of dietary feedback control of HMG-CoA reductase in hepatomas is unclear. One possibility is that in the hepatomas there is an increased proportion of HMG-CoA reductase in the active form or there is an abnormal phosphorylation mechanism.

The study of this mechanism *in vitro* requires considerable care since artifacts can be produced due to the presence of mevalonate kinase and ATP(Mg) in the assay causing a loss of radio-labelled mevalonate. The

conditions used in this work exclude any influence of mevalonate kinase activity in the measurement of HMG-CoA reductase inactivation.

The data presented show that the percentage of active enzyme in the hepatoma is similar to that of the normal and host liver *in vivo*. These data also imply that the phosphatase responsible for activation of HMG-CoA reductase during isolation is present in all three tissues. Reductase kinase activity is also present in host liver and hepatoma 5123C. The half times of inactivation in the three tissues are very similar. This indicates that the ratio of HMG-CoA reductase activity to reductase kinase activity in these tissues is similar since the initial HMG-CoA reductase activity in the hepatoma was 8 fold that in the host liver.

These studies indicate that the phosphorylation/dephosphorylation of HMG-CoA reductase occurs in hepatomas. However, abnormalities in other parts of the proposed reversible phosphorylation mechanism (1, 2, 3, 4) cannot be excluded.

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