REVERSIBLE MODULATION OF RAT LIVER 3-HYDROXY 3-METHYL GLUTARYL COENZYME A REDUCTASE

Evidence for an enzyme-catalyzed phosphorylation–dephosphorylation system

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1. Introduction

3-Hydroxy 3-methyl glutaryl coenzyme A reductase (Mevalonate:$NADP^+$ oxidoreductase (CoA acylating) EC 1.1.1.34) is commonly considered the major regulatory step in the cholesterol biosynthetic pathway. This regulation may be achieved on one hand through the number of enzyme molecules present in liver, the concentration of which is changed by cholesterol feeding. On the other hand, more rapid modulation in HMG-CoA Rd activity under different circumstances has been reported. A cytosol fraction from rat liver could inactivate the microsomal enzyme in the presence of ATP-Mg [1]. HMG-CoA Rd from rat liver was inactivated by a protein obtained from human fibroblasts in the presence of ATP-Mg [2]. HMG-CoA Rd from mouse liver is activated by preincubating liver homogenates at 37°C [3,4].

We describe here experiments showing reversible interconversion of HMG-CoA Rd between an active and an inactive form. If [γ-^{32}P]ATP is used the changes of the enzyme activity correlate well with changes in the amount of ^{32}P incorporated into protein. This points to a phosphorylation–dephosphorylation mechanism to be involved in the regulation of HMG-CoA Rd.

Abbreviations: HMG-CoA Rd, 3-hydroxy 3-methyl glutaryl coenzyme A reductase; cAMP, adenosine 3':5' cyclic monophosphate; DTT, dithiothreitol; TCA, trichloroacetic acid

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2. Materials and methods

DL-hydroxy methyl [3-^{14}C]glutaryl CoA (spec. act. 1.2×10^4 cpm/nmol) was prepared from DL-hydroxy methyl [3-^{14}C]glutaric acid (New England Nuclear, Dreieichenhain) and CoA (Sigma Chem Co., St Louis, MO) according to the method in [5]. The product was determined as in [6]. [γ-^{32}P]ATP was prepared from unlabelled ATP (Sigma) and ^{32}P (Amersham Ltd, Buckinghamshire) as in [7]. Glycerol kinase from *Escherichia coli* was from Sigma. HF Silica Gel plates and usual chemicals were from Merck, Darmstadt.

Wistar rats fed ad libitum with laboratory chow were anesthesized with ether, and sacrificed at midnight. Livers were excised and homogenized 1:3, w/v, using a tight-fitting Teflon pestle in a medium consisting of 0.1 M sucrose, 0.05 M KCl, 0.04 M potassium phosphate, 0.03 M EDTA and 0.01 M DTT, pH 7.2 (buffer 1). The homogenate was centrifuged for 20 min at 30 000 × *g* at 4°C. The resulting supernatant was recentrifuged at 105 000 × *g* for 90 min. The microsomal pellet was resuspended in EDTA free buffer 1 and washed and centrifuged twice more. The washed pellets were finally stored at −40°C until used. The enzyme activity remained constant for weeks.

For the activation experiments, homogenates

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(1:3) were prepared from fresh rat livers in a medium consisting of 0.1 M sucrose, 0.05 M KCl, and 0.04 M Tris–HCl, pH 7.2 (buffer 2), centrifuged at 30 000 × *g* for 20 min. The resulting supernatant was further centrifuged at 105 000 × *g* for 90 min and the supernatant was used as the source of activation factor. The temperature was kept at 4°C through all the procedures.

The preincubation medium with total vol. 0.5 ml contained 40 μmol Tris–HCl, 10 μmol $MgCl_2$, 1 μmol EDTA, 10 μmol mercaptoethanol, 3 μmol ATP and 4–5 mg microsomal protein, pH 7.2. Incubation was terminated by addition of 15 μl 0.6 M EDTA. In the reactivation experiments, ATP was first destroyed by adding 0.05 ml mixture containing 3 μmol glycerol and 5 units glycerol kinase. After 1 min the reaction was started by addition of an appropriate amount of 105 000 × *g* supernatant containing 12 mg protein.

HMG-CoA Rd activity was measured as in [4], starting the reaction by addition of 0.2–0.4 mg microsomal protein from the preincubation mixture. The labelled mevalonolactone produced during the reaction was isolated by thin-layer chromatography [8] using 150 μl reaction mixture. Mevalonolactone was visualized in the plate by exposure to ultraviolet light at 366 nm. The shaded area was scrapped out and counted in a liquid scintillation counter for ^{14}C using Bray's solution. Unless otherwise indicated, temperatures were: 30°C for preincubation; 37°C for the enzyme assay. Protein was determined as in [9].

In experiments using [γ-^{32}P]ATP, aliquots of the preincubation mixture were placed on 2X2 cm Whatman 3 filter paper and introduced in boiling 10% trichloroacetic acid (TCA). The papers were washed 3 times in hot 10% TCA, finally dried with ether and counted for ^{32}P according to method B of [10]. For the purpose of this paper, we have defined 1 unit of HMG-CoA Rd as that activity which converts 1 nmol HMG-CoA into mevalonate in 1 h at 37°C under the standard assay conditions.

3. Results

When washed rat liver microsomes were preincubated with ATP-Mg, the HMG-CoA Rd activity was rapidly reduced reaching 5% initial activity within 20 min (fig.1). This time-dependent inactivation

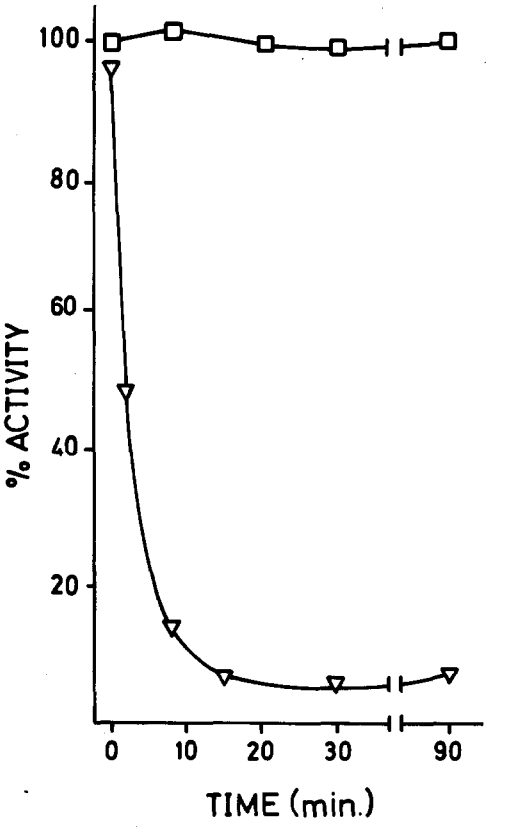

Fig.1. Inactivation of microsomal HMG-CoA Rd. Twice washed microsomes were preincubated at 30°C at different times with (▽–▽) or without (□–□) 6 mM ATP, 20 mM $MgCl_2$. The reaction was arrested with EDTA, and the HMG-CoA Rd activity was measured at the indicated times. % initial value (15.0 units/mg protein) is represented on the ordinate.

was not observed when ATP, Mg^{2+} or both were omitted or when EDTA was added prior to preincubation. cAMP in the concentration range of 10^{-2}–10^{-7} M did not alter the rate of inactivation.

The dependence of the inactivation reaction upon ATP concentration is illustrated in fig.2. The molar ratio ATP/Mg was kept constant at 1:3.3 throughout the experiments. When the initial rates of inactivation (% inactivation/min preincubation) were plotted against the ATP concentration, an hyperbolic profile was obtained. By double reciprocal plots the calculated $S_{0.5}$ value for ATP was 1.2 mM. The inactivation of the enzyme remained unchanged when ATP was removed by centrifugation at 105 000 × *g*.

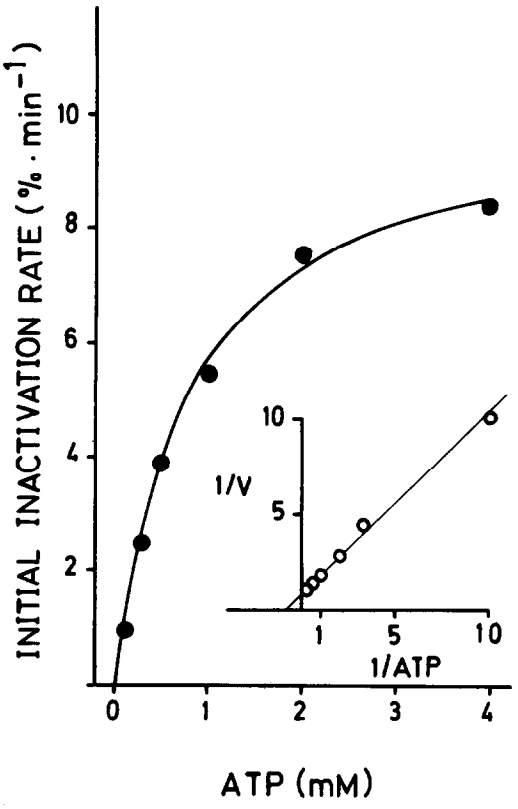

Fig.2. Dependence of the HMG-CoA Rd inactivation on ATP concentration. Ordinates represent initial rates of inactivation (% inactivation in 1 min). ATP concentrations were varied as indicated in the abscissa. ATP/Mg ratio was kept constant at 1/3.3. Temperature was maintained at 15°C.

In order to study the reversibility of this inactivation, 2 series of tubes containing twice-washed microsomes were first incubated with ATP under standard conditions. After 20 min, ATP was destroyed as described before by adding glycerol–glycerol kinase. Fresh 105 000 × *g* supernatant, 0.4 ml, was then added to the first series whereas buffer 2 was added to the second series. As shown in fig.3 there was a dramatic recuperation of enzyme activity after addition of the supernatant fraction which within 30 min reached values even higher than initially, and similar to those obtained when no inactivated microsomes were incubated for 30 min with 0.4 ml of 105 000 × *g* supernatant. No effect was observed in the control where the supernatant fraction was replaced by buffer only. Boiling the 105 000 × *g* supernatant prior to addition prevented the reactivation. Fluoride was capable of completely suppresing the reactivation activity of the 105 000 × *g* supernatant. Furthermore when maximal reactivation was reached the enzyme could be inactivated again by the addition of ATP-Mg plus fluoride to the incubation mixture (fig.4).

To explore the mechanism responsible for the inactivation–reactivation of HMG-CoA Rd, experiments were performed using [γ-^{32}P]ATP. The ^{32}P incorporation into 10% TCA-precipitable fraction was measured as well as the HMG-CoA Rd activity in these studies.

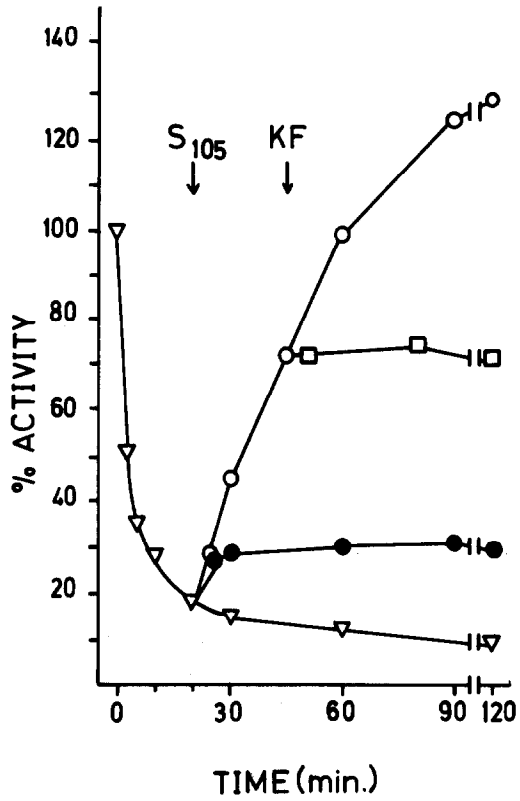

Fig.3. Inactivation–reactivation profile of microsomal HMG-CoA Rd. Twice washed microsomes were preincubated under the same conditions as in fig.1. At 20 min, a glycerol–glycerol kinase mixture was added to one series of tubes (•–•) whereas to another series 0.4 ml 105 000 × *g* supernatant corresponding to 12 mg protein was added as well (○–○). To some tubes of these last series, KF was added at 45 min to give final conc. 50 mM (□–□). The HMG-CoA Rd activity was measured at the times indicated and % initial value (17.2 units/mg protein) is represented on the ordinate.

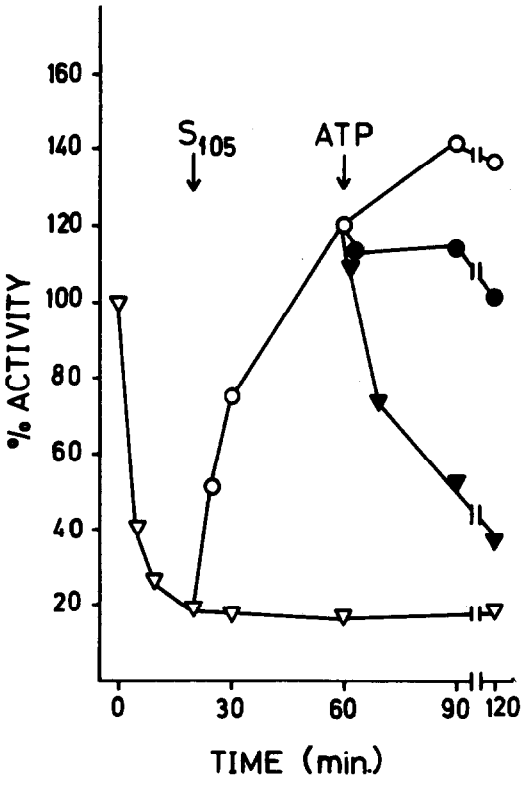

Fig.4. Inactivation–reactivation–inactivation profiles of microsomal HMG-CoA Rd. Twice washed microsomes were preincubated with ATP-Mg as described in fig.1. At 20 min, a glycerol–glycerol kinase mixture and thereafter 0.4 ml 105 000 × *g* supernatant corresponding to 12 mg protein was added as well (○–○). At 60 min KF at a final concentration of 50 mM was added to one series of tubes (●–●) and to the other series, KF plus 6 mM ATP, 20 mM $MgCl_2$ was added (▼–▼). The reaction was arrested cooling in an ice bath, and by addition of 15 μl 0.6 M EDTA, and the HMG-CoA Rd activity was immediately measured. % initial value (12.2 units/mg protein) is represented on the ordinate.

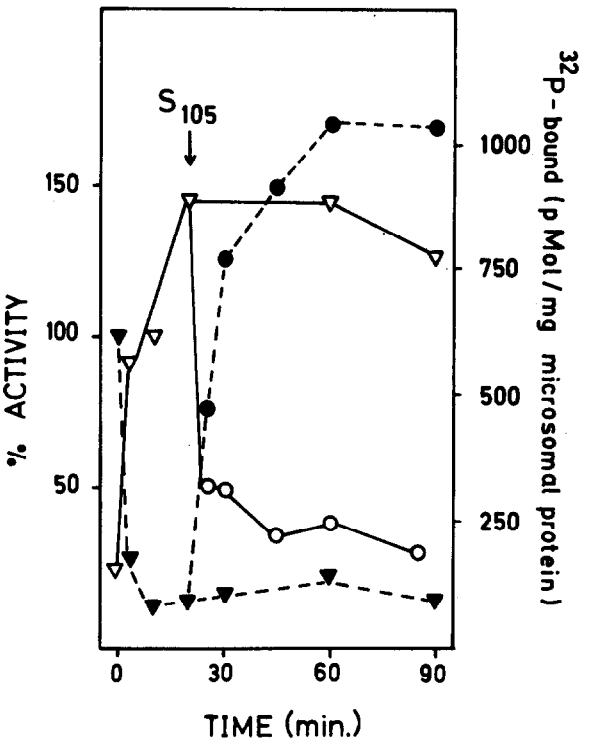

Fig.5. Inactivation–reactivation cycle of microsomal HMG-CoA Rd in the presence of 6 mM [γ-^{32}P]ATP (spec. act. 1.41 × 10^7 cpm/μmol), 20 mM $MgCl_2$. Twice washed microsomes were preincubated as described in fig.3 except that radioactive ATP was used. The HMG-CoA Rd activity was measured and % initial value (15.6 units/mg protein) are represented on the left ordinate (closed symbols). The ^{32}P bound to microsomal protein was measured as in section 2, and the pmol ^{32}P/mg protein are represented on the right ordinate (open symbols).

As illustrated in fig.5 there was an increasing uptake of ^{32}P into protein concomitant with the fall in HMG-CoA Rd activity. Conversely, when the supernatant was added, a decrease in protein-bound radioactivity correlated with the restoration of enzyme activity. Furthermore, when fluoride 50 mM was added to suppress the reactivation process, both the radioactivity and the HMG-CoA Rd activity remained unchanged during the preincubation period.

4. Discussion

The experiments described here clearly show that incubation of rat liver microsomes in the presence of ATP-Mg leads to almost complete inactivation of HMG-CoA Rd within 20 min. At variance with the results in [1,11] we find that no cytosolic fraction is necessary under our experimental conditions to produce the inactivation. ATP-Mg-dependent inhibition of microsomal HMG-CoA Rd was reported [12] but this was variable in degree and not consistently reproducible ranging from zero to complete. Instead,

the percentage of inactivation in our experiments was consistent and ranged from 85–95% in 20 min.

Our studies furthermore show that the 105 000 × *g* supernatant of rat liver contains a heat labile factor that can reactivate the inactivated enzyme. The reactivated enzyme can be again inactivated by ATP if fluoride is also present which inhibits the activity of the reactivation factor of the supernatant fraction. Our results strongly suggest that rat liver HMG-CoA Rd belongs to the class of interconvertible enzymes which is inactivated by ATP-dependent phosphorylation and reactivated by dephosphorylation.

Our data make it very likely that there is a microsome-bound kinase which catalyses the inactivation reaction whereas a phosphatase present in the 105 000 × *g* supernatant is responsible for the reactivation of the enzyme. Though supporting this view, our experiments with [γ-^{32}P]ATP may not appear so strictly convincing as other protein phosphorylation and dephosphorylation reactions not related to HMG-CoA Rd which may occur in microsomes [13]. However, the close correspondence of ^{32}P incorporation and inactivation on the one hand and of ^{32}P release and reactivation on the other, offers rather good evidence for an interrelationship of the two processes. Whether the phosphorylation and dephosphorylation reactions concern the HMG-CoA Rd molecule directly or involve the cooperation of other proteins must remain open. This and other questions concerning the mechanisms of the regulation of HMG-CoA Rd interconversion which appears independent of cAMP are under current investigation.

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