Differences in mechanisms of modulation between rat liver cholesterol 7α-hydroxylase and HMG-CoA reductase

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The effects of microsomal HMG-CoA reductase kinase, cytosolic phosphoprotein phosphatase and cytosolic, thiol-dependent cholesterol 7α-hydroxylase stimulatory protein on purified cholesterol 7α-hydroxylase and HMG-CoA reductase from rat liver were compared. Neither HMG-CoA reductase kinase nor phosphoprotein phosphatase had any significant effect on cholesterol 7α-hydroxylase activity. They inhibited and stimulated, respectively, the activity of HMG-CoA reductase. The purified cytosolic protein which stimulated cholesterol 7α-hydroxylase threefold in the presence of glutathione had no effect on HMG-CoA reductase. The results show that there are separate intracellular systems for modulation of cholesterol 7α-hydroxylase and HMG-CoA reductase.

1. INTRODUCTION

Cholesterol 7α-hydroxylation is the initial and major rate-limiting step in the conversion of cholesterol into bile acids [1]. The reaction is catalyzed by a microsomal monooxygenase system involving cytochrome P-450 and NADPH-cytochrome P-450 reductase [2]. Sulfhydryl groups are important for the catalytic activity [3] and the modulation of cholesterol 7α-hydroxylase by a cytosolic, thiol-dependent protein has been recently reported [4].

Contradictory reports have appeared concerning the existence of a phosphorylation-dephosphorylation mechanism for regulation of cholesterol 7α-hydroxylase [4–8]. Reversible phosphorylation is involved in short-term regulation of another microsomal enzyme, HGM-CoA reductase, catalyzing the rate-limiting step in cholesterol biosynthesis [9,10]. Cholesterol 7α-hydroxylase and HMG-CoA reductase are in many instances re-gulated by the same factors, including hormonal and nutritional status as well as the concentration of bile acids in portal blood [1]. In addition, it has been recently reported that sulfhydryl groups are important for catalytic activity also of HMG-CoA reductase [11]. It should therefore be of interest to compare the effects of HMG-CoA reductase kinase, phosphoprotein phosphatase and cytosolic, thiol-dependent cholesterol 7α-hydroxylase stimulatory protein on the two rate-limiting enzymes. The present communication reports such a study with purified cholesterol 7α-hydroxylase and HMG-CoA reductase from rat liver microsomes.

2. MATERIALS AND METHODS

2.1. Materials

DL-3-hydroxy-3-[*methyl*-³H]methylglutaryl-CoA was obtained from New England Nuclear (12.2 Ci/mol), [4-¹⁴C]cholesterol (61 Ci/mol) was obtained from the Radiochemical Center, Amersham, England. Sephadex G-100 and Sephadex G-75 were from Pharmacia.

Abbreviation: HGM-CoA, 3-hydroxy-3-methylglutaryl coenzyme A

2.2. Methods

Cytochrome P-450 active in cholesterol 7α-hydroxylation was prepared from liver microsomes of cholestyramine-treated rats as described [12].

NADPH-cytochrome P-450 reductase (spec. act. 50 units per mg) was prepared from phenobarbital-treated rats according to Yasukochi and Masters [13].

HMG-CoA reductase was prepared from liver microsomes of cholestyramine-treated rats, was solubilized as described by Edwards et al. [14] and precipitated with ammonium sulfate (35–50%) [15].

Phosphoprotein phosphatase from liver of untreated rats was prepared as described by Titanji [16], with the exception that the $100000 \times g$ supernatant was used as starting material instead of the $16000 \times g$ supernatant. Sephadex G-100 chromatography was used instead of Sepharose 6B chromatography in the last purification step.

Microsomal HMG-CoA reductase kinase was prepared from untreated rats according to Ferrer and Hegardt [17].

Cytosolic cholesterol 7α-hydroxylase stimulatory protein was purified as described by Danielsson et al. [4] with the exception that the isoelectric focusing step was replaced by Sephadex G-75 chromatography. The protein obtained after this modification of the purification procedure showed one protein band ($M_r = 25000$) upon polyacrylamide gel electrophoresis in the presence of SDS.

2.3. Incubation procedures and analyses of incubation mixtures

Incubations with cholesterol were performed at 37°C for 20 min in a total volume of 0.75 ml of Tris-acetate buffer, pH 7.4, containing 20% glycerol. The incubation mixture contained 0.1 nmol cytochrome P-450, 0.5 units NADPH-cytochrome P-450 reductase, 15 μg dilauroylglycero-3-phosphorylcholine, 0.3 mg Triton X-100 in 20 μl water, 0.5 μmol reduced glutathione or 2 μmol dithiothreitol, 25 nmol cholesterol added in 15 μl acetone, and 0.9 μmol NADPH. The incubations were terminated and analyzed for cholesterol 7α-hydroxylase activity as described [2].

Incubations with HMG-CoA reductase were performed at 37°C for 20 min in a total volume of 150 μl of 50 mM Tris-Cl buffer, pH 6.8, containing 0.2 M KCl. The incubation mixture contained 95 μg HMG-CoA reductase, 0.75 μmol dithiothreitol, 50 nmol HMG-CoA and 0.45 μmol NADPH. The incubations were terminated by addition of 25–50 μl of 10 M HCl, shaken vigorously and incubated for 20 min at 37°C to allow complete lactonization of mevalonic acid. HMG-CoA reductase activity was assayed by the mixed-phase assay described by Philipp and Shapiro [18].

In experiments on the effect of phosphoprotein phosphatase on cholesterol 7α-hydroxylase and HMG-CoA reductase, appropriate amounts of phosphatase were preincubated at 37°C for 60 min with cytochrome P-450 or HMG-CoA reductase and 0.75 μmol dithiothreitol in a volume of 95 μl of 50 mM Tris-Cl buffer, pH 6.8, containing 0.2 M KCl. After preincubation, 11 μmol NaF were added together with cofactors and substrates and the activities were analyzed as described above.

In experiments on the effect of HMG-CoA reductase kinase, cytochrome P-450 or HMG-CoA reductase were preincubated with appropriate amounts of kinase as described for homogeneous HMG-CoA reductase by Ferrer and Hegardt [17]. Cytochrome P-450 or HMG-CoA reductase, kinase, 0.45 μmol dithiothreitol, and 20 μl of a $6:20$ mM ATP/MgCl$_2$ solution were preincubated at 37°C for 60 min. After preincubation, 20 μl of 40 mM KH$_2$PO$_4$ buffer, pH 7.2, containing 25 mM sucrose, 100 mM NaF, 30 mM EDTA, and 1 mM dithiothreitol, were added with the other components and the activities were analyzed as described above.

In experiments on the effect of purified cytosolic cholesterol 7α-hydroxylase stimulatory protein on cholesterol 7α-hydroxylase and HMG-CoA reductase, appropriate amounts of cytosolic protein were first preincubated with 0.1 μmol dithiothreitol for 20 min and then with glutathione and cytochrome P-450 or HMG-CoA reductase for 20 min at 37°C in a volume of 150 μl Tris-acetate buffer, pH 7.4, containing 20% glycerol. After preincubation, cofactors and substrate were added to cholesterol incubations as described above. To make incubations with HMG-CoA reductase comparable to those with cholesterol 7α-hydroxylase, 0.12 mg Triton X-100 in water, 6 μg dilauroylglycero-3-phosphorylcholine, 100 nmol HMG-CoA and 0.6 μmol NADPH were added to a final

volume of 260 μl. Incubations were run at 37°C for 20 min and were terminated and analyzed for HMG-CoA reductase activity as described above.

3. RESULTS

Cytochrome P-450 catalyzing 7α-hydroxylation of cholesterol and HMG-CoA reductase were incubated with microsomal HMG-CoA reductase kinase, cytosolic phosphoprotein phosphatase and cytosolic cholesterol 7α-hydroxylase stimulatory protein under the same conditions. Fig.1 shows that preincubation of increasing amounts of microsomal HMG-CoA reductase kinase and ATP/MgCl$_2$ with cholesterol 7α-hydroxylase had no effect on the 7α-hydroxylation of cholesterol. The HMG-CoA reductase kinase had the expected inactivating effect on HMG-CoA reductase. A 90% loss of activity was seen in the presence of 50 μg kinase and ATP/MgCl$_2$.
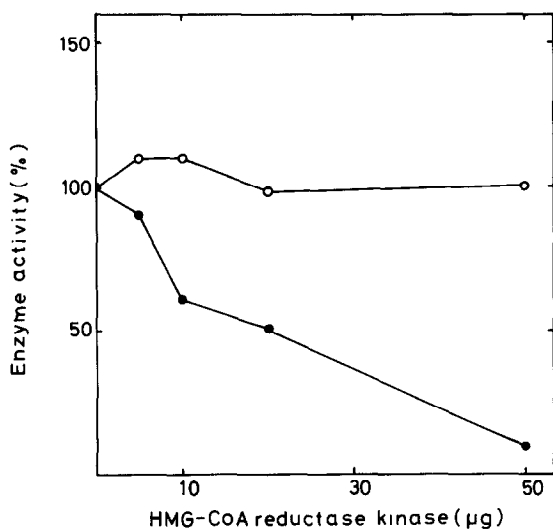
Fig.2 shows that preincubation of increasing amounts of phosphoprotein phosphatase with cholesterol 7α-hydroxylase had no effect on 7α-hydroxylation of cholesterol. Increasing amounts of phosphoprotein phosphatase in the preincubation mixture stimulated HMG-CoA reductase twofold and then remained constant. Maximal stimulation was obtained with 5 μg of phosphatase.

Fig.3A shows that preincubation of cholesterol 7α-hydroxylase stimulatory protein (50 μg) with cholesterol 7α-hydroxylase and increasing concentrations of glutathione stimulated the conversion of cholesterol into 7α-hydroxycholesterol. A threefold stimulation was obtained with 0.5–1 mM glutathione. The protein had no stimulatory effect in the absence of glutathione. As reported [4] glutathione also stimulated cholesterol 7α-hydroxylation in the absence of stimulatory protein. However, the degree of stimulation was less
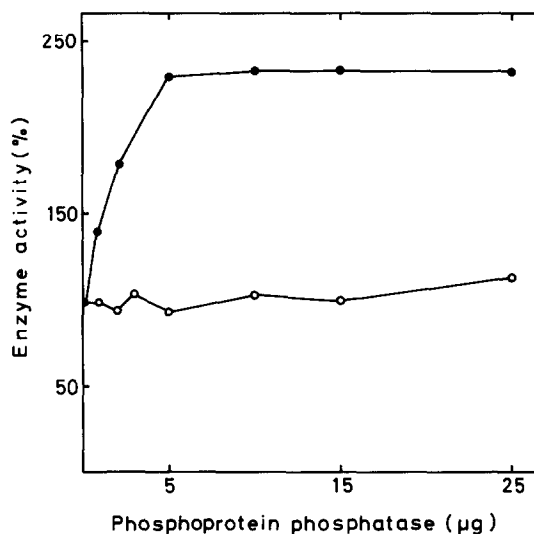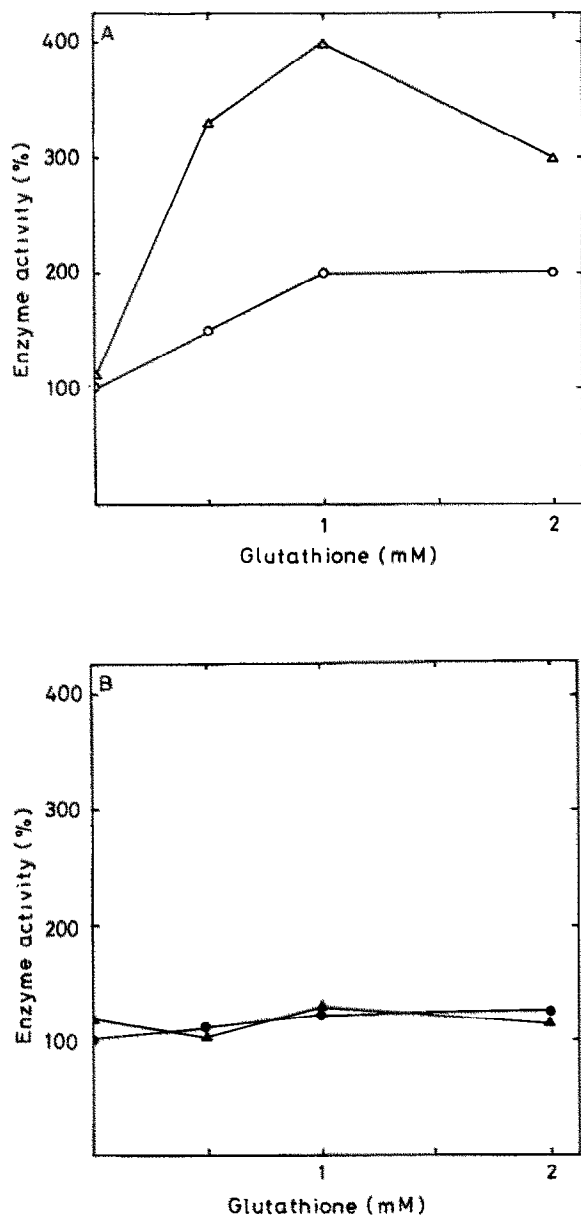

Fig.1. Effect of HMG-CoA reductase kinase on cholesterol 7α-hydroxylase and HMG-CoA reductase activities. Incubations were performed in the presence of ATP and MgCl$_2$ as described in section 2. (O) Cholesterol 7α-hydroxylase activity; (●) HMG-CoA reductase activity. Activities are expressed as percentages of control values. 100% cholesterol 7α-hydroxylase activity corresponds to 285 pmol 7α-hydroxycholesterol formed per nmol cytochrome P-450 and min. 100% HMG-CoA reductase activity corresponds to 2.25 nmol mevalonic acid formed per mg protein and min.

Fig.2. Effect of phosphoprotein phosphatase on cholesterol 7α-hydroxylase and HMG-CoA reductase activities. Incubation conditions were as described in section 2. (O) Cholesterol 7α-hydroxylase activity; (●) HMG-CoA reductase activity. Activities are expressed as percentages of control values. 100% cholesterol 7α-hydroxylase activity corresponds to 290 pmol 7α-hydroxycholesterol formed per nmol cytochrome P-450 and min. 100% HMG-CoA reductase activity corresponds to 2.25 nmol mevalonic acid formed per mg protein and min.

Fig.3. (A) Effect of reduced glutathione and purified cytosolic cholesterol 7α-hydroxylase stimulatory protein on cholesterol 7α-hydroxylase activity. Incubation conditions were as described in section 2. In experiments on the effect of purified cytosolic protein, 50 μg of this fraction were included in the incubation mixture. (O) Reduced glutathione; (Δ) reduced glutathione in presence of cytosolic protein. Cholesterol 7α-hydroxylase activity is expressed as percentage of control values. 100% activity corresponds to 255 pmol 7α-hydroxycholesterol formed per nmol cytochrome P-450 and min. (B) Effect of reduced glutathione and purified cytosolic cholesterol 7α-hydroxylase stimulatory protein on HMG-CoA reductase activity. Incubation conditions were as described in section 2. In experiments on the effect of purified cytosolic protein, 50 μg of this fraction were included in the incubation mixture. (●) Reduced glutathione; (▲) reduced glutathione in presence of cytosolic protein. HMG-CoA reductase activity is expressed as percentage of control values. 100% HMG-CoA reductase activity corresponds to 1.2 nmol of mevalonic acid formed per mg protein and min.

4. DISCUSSION

Several recent reports in which whole microsomes have been used as enzyme source suggest that cholesterol 7α-hydroxylase is modulated by a reversible phosphorylation mechanism [5–7]. The present results show that the activity of purified, reconstituted cholesterol 7α-hydroxylase system is not modulated by a kinase and a phosphatase active on HMG-CoA reductase. Thus, it appears less likely that cholesterol 7α-hydroxylase is regulated by a phosphorylation-dephosphorylation mechanism. However, the results do not exclude the existence of other protein kinases and phosphatases which act upon and even may be specific for cholesterol 7α-hydroxylase.

The cytosolic cholesterol 7α-hydroxylase stimulatory protein had no effect on HMG-CoA reductase in presence of glutathione concentrations (0.5–2 mM) resulting in stimulation of cholesterol 7α-hydroxylase. As reported, glutathione alone had a stimulatory effect on cholesterol 7α-hydroxylase although smaller than that observed in the presence of the stimulatory protein. Under the same conditions, glutathione alone had

than in the presence of the stimulatory protein. Fig.3B shows that preincubation of HMG-CoA reductase with cholesterol 7α-hydroxylase stimulatory protein and glutathione had no effect on HMG-CoA reductase activity.

no effect on HMG-CoA reductase activity. It should be mentioned that HMG-CoA reductase is stimulated by glutathione in concentrations 10-fold higher (10–20 mM) than those required for stimulation of cholesterol 7α-hydroxylation [11].

The results of the present communication indicate that short-term regulation of rat liver cholesterol 7α-hydroxylase and HMG-CoA reductase does not involve the same type of mechanism and that there are separate intracellular modulatory systems for these two regulatory enzymes in cholesterol metabolism.

ACKNOWLEDGEMENTS

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