Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 212-219

REGULATION OF HYDROXYLATIONS IN BIOSYNTHESIS OF BILE ACIDS: MODULATION OF RECONSTITUTED 12α-HYDROXYLASE ACTIVITY BY PROTEIN FRACTIONS FROM RABBIT LIVER CYTOSOL AND MICROSOMES

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Received April 4, 1983

The activity of purified 12α-hydroxylase from rabbit liver microsomes was modulated by including protein fractions from rabbit liver microsomes and cytosol into the system. The microsomal protein fraction stimulated 12α- hydroxylation two times. The cytosolic fraction inhibited the reaction markedly. The microsomal 12α-hydroxylase stimulatory activity was labile and the cytosolic 12α-hydroxylase inhibitory activity was stable to mild heat treatment. Addition of ATP and $MgCl_2$ or NaF had no effect on the activities of the two protein fractions. The activity of the microsomal stimulatory fraction decreased upon storage but could be reactivated by addition of reduced glutathione to the system.

The conversion of cholesterol into cholic acid requires a steroid 12α-hydroxylation in the microsomal fraction of the liver (1). The 12α-hydroxylase activity is influenced by starvation (2), administration of bile acids (3) and thyroid hormone (4). The 12α-hydroxylase system has been ascribed a role in the regulation of bile acid formation (1).

Several groups of investigators have reported recently that partially purified protein fractions from rat liver cytosol influence the activity of microsomal cholesterol 7α-hydroxylase, the rate-limiting enzyme system in bile acid biosynthesis (5-8). It is likely that modulatory proteins are involved also in the regulation of the activity of other key enzymes in cholesterol metabolism, such as steroid 12α-hydroxylase.

The 12α-hydroxylation reaction is catalyzed by a monooxygenase system involving a cytochrome P-450 and NADPH-cytochrome P-450 reductase (9,10). In rabbit liver microsomes the 12α-hydroxylase

0006-291X/83 $1.50

activity is present in apparently homogenous cytochrome P-450 LM_4 (10).

The present communication reports the isolation of a microsomal and a cytosolic protein fraction from rabbit liver that modulate 5ß-cholestane-3α,7α-diol 12α-hydroxylase activity of cytochrome P-450 LM_4.

EXPERIMENTAL PROCEDURE

Materials. 5ß [7ß-^{3}H] Cholestane-3α,7α-diol (500 Ci/mol) was prepared as described previously (11). [4 -^{14}C] Cholesterol (61 Ci/mol) was obtained from the Radiochemical Centre, Amersham, England. DEAE-cellulose, Triton X-100, dilauroyl-glycero-3-phosphorylcholine and cofactors were obtained from Sigma Chemical Co. DEAE-cellulose (DE-52) was obtained from Whatman.

Methods. Untreated, phenobarbital-treated (10) or cholestyramine-treated (11) male rabbits of the New Zealand strain weighing about 2 kg were used. The microsomal fraction was prepared from a 33% (w/v) liver homogenate in 0.25 M sucrose containing 1 mM EDTA and 5 mM Tris-Cl, pH 7.4 (11). The 100,000 x g supernatant fluid was used as starting material for purification of cytosolic protein. Protein in preparations of cytochrome P-450 and NADPH-cytochrome P-450 reductase was determined as described by Lowry et al. (12). Protein in preparations of microsomal 12α-hydroxylase stimulator and cytosolic 12α-hydroxylase inhibitor was measured as described by Bradford (13).

Preparation of microsomal 12α-hydroxylase stimulator. Liver microsomes from untreated rabbits were suspended in 100 mM potassium pyrophosphate buffer, pH 7.4, and centrifuged at 100,000 x g for 1 h. The washed microsomes were suspended in 10 mM potassium phosphate buffer, pH 7.4, to a protein concentration of 16 mg per ml. The suspension was sonicated for a total period of 3 min at intervals of 15 s using a Megason sonifier at full output. The sonicated sample was centrifuged at 100,000 x g for 1 h. The 100,000 x g precipitate was resuspended in 10 mM potassium phosphate buffer, sonicated at intervals of 15 s for a total period of 6 min and centrifuged at 100,000 x g for 1 h. The supernatant fluid obtained was applied to a DE-52 cellulose column (3 x 20 cm), equilibrated with 10 mM potassium phosphate buffer, pH 7.4. Protein fractions with stimulatory activity were eluted in one peak with the equilibrating buffer. The fractions with maximal absorbance at 280 nm were pooled and used as microsomal 12α-hydroxylase stimulator.

Preparation of cytosolic 12α-hydroxylase inhibitor. Liver cytosol from phenobarbital-treated rabbits was fractionated with polyethylene glycol 6000 (14). The protein fraction precipitating between 6 and 12% (w/v) polyethylene glycol was collected by centrifugation at 20,000 x g for 15 min. The precipitate was dissolved in 50 mM potassium phosphate buffer, pH 7.5, containing 0.25 M sucrose, and dialyzed against the same buffer. The dialyzed sample was applied to a DEAE-cellulose column (3 x 20 cm), equilibrated with the dialysis buffer. The column was washed with the equilibrating buffer until the absorbance at 280 nm of the eluate was below 0.1. Protein was then eluted with a linear gradient of potassium chloride (0 to 0.6 M) in 600 ml of the equilibrating buffer. The fractions containing 12α-hydroxylase

inhibitory activity were pooled, concentrated in a Diaflo ultrafiltration cell (Amicon PM-10 filter) and dialyzed against 50 mM potassium phosphate buffer, pH 7.5, containing 0.25 M sucrose.

Preparation of cytochrome P-450 and NADPH-cytochrome P-450 reductase. Cytochrome P-450 LM_4 fraction was prepared as described by Coon and associates (14) from cholestyramine-treated rabbits. The preparation contained 11 nmol of cytochrome P-450 per mg of protein. Cytochrome P-450 was determined as described by Omura and Sato (15).

NADPH-cytochrome P-450 reductase was prepared from phenobarbital-treated rabbits as described by Yasukochi and Masters (16) and had a specific activity of 50 units per mg of protein.

Incubation procedures and analyses of incubation mixtures. Incubations with 5β-cholestane-3α,7α-diol, 125 nmol, were performed at 37°C for 20 min with 0.25 nmol of cytochrome P-450 LM_4, 1.5 units of NADPH-cytochrome P-450 reductase, 15 μg of dilauroylglycero-3-phosphorylcholine, 0.9 μmol of NADPH and appropriate amount of cytosolic or microsomal subfractions in a total volume of 0.75 ml of 150 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA.

Incubations with cholesterol, 25 nmol in 15 μl of acetone, were performed as described for 5β-cholestane-3α,7α-diol with the exception that 50 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol was used instead of the 150 mM potassium phopshate buffer and that 0.3 mg Triton X-100 in 20 μl of water and 5 mM dithiotreitol were added to the incubation mixture.

The incubations were terminated and analyzed as described previously (11).

RESULTS

Microsomal 12α-hydroxylase stimulator. A protein fraction which had a stimulatory effect on reconstituted 12α-hydroxylase activity was prepared by repeated sonication of washed microsomes followed by DEAE-cellulose chromatography of the sonicate. Stimulatory activity was eluted with the equilibration buffer in chromatography of both the first and second sonicate. However, the amount of protein required for a two-fold stimulation of 12α-hydroxylase activity was about twenty times lower with the preparation from the second sonicate. This fraction was therefore used as the microsomal 12α-hydroxylase stimulator in the present study (cf. Fig. 1). The stimulatory protein fraction contained no measurable cytochrome P-450 or cytochrome b_5 (17) and did not show NADPH-cytochrome c reductase activity. Fig. 1 shows that the 12α-hydroxylase stimulatory activity was linear with the amount of protein up to a certain level and then remained constant. Maximal stimulation was obtained with 2 μg of protein. The protein fraction showed upon SDS-polyacrylamide gel electrophoresis a major band with apparent M_r of around 60,000 and several minor bands. Table 1 shows that the stimulatory activity was not dependent on ATP or $MgCl_2$ and was not affected by NaF.

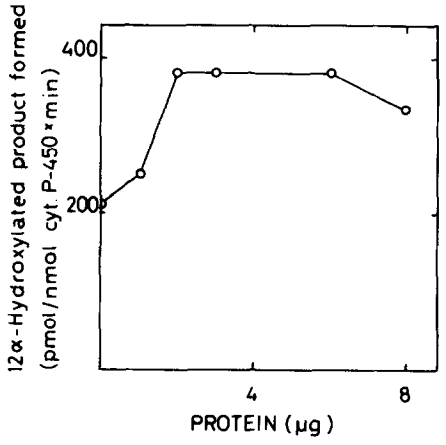

Fig. 1. Effect of microsomal protein fraction on stimulation of reconstituted 12α-hydroxylase activity. Incubation conditions were as in Table 1 except for the varying concentrations of microsomal protein fraction.

The activity was destroyed upon heat treatment at 80°C for 5 min. It was observed that the stimulatory activity decreased after storage of the fractions for only a few days at -20°C. Table 2 shows the effect of reduced glutathione and glutathione disulfide on a 12α-hydroxylase stimulatory fraction which had lost most of its stimulatory activity during storage. Addition of reduced glutathione to the incubation mixture restored the

TABLE 1. Effect of ATP, $MgCl_2$ and NaF on protein fractions modulating 12α-hydroxylase activity.

System	12α-hydroxylation in presence of		
	No addition	ATP $MgCl_2$	NaF
	pmol/nmol cytochrome P-450 x min		
Control	201	160	147
Control + 12α-hydroxylase stimulator	388	317	382
Control + 12α-hydroxylase inhibitor	45	40	47

The control system contained 0.25 nmol of cytochrome P-450 LM_4, 1.5 units of NADPH-cytochrome P-450 reductase, 15 μg of dilauroylglycero-3-phosphorylcholine, 0.9 μmol of NADPH and 125 nmol of 5ß-cholestane-3α,7α-diol in a total volume of 0.75 ml of 150 mM potassium phopshate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. Incubations were performed at 37°C for 20 min. In experiments on the effects of stimulatory protein fraction (2 μg) and inhibitory protein fraction (0.4 mg) these fractions were included in the incubation mixture. The concentrations of ATP, $MgCl_2$ and NaF were, 2 mM, 4 mM and 50 mM respectively.

TABLE 2. Effect of glutathione and glutathione disulfide on protein fractions modulating 12α-hydroxylase activity.

System	12α-hydroxylation in presence of		
	No addition	GSH[a]	GSSG
	pmol/nmol cytochrome P-450 x min		
Control	115	90	100
Control + 12α-hydroxylase stimulator	165	327	107
Control + 12α-hydroxylase inhibitor	38	35	54

Incubation conditions and amount of protein added to the incubation mixture were the same as in Table 1. The concentrations of reduced glutathione and glutathione disulfide were 2.5 mM and 0.2 mM, respectively.

a The following abbreviations are used: GSH, reduced glutathione; GSSG, glutathione disulfide.

12α-hydroxylase stimulatory activity of the fraction whereas glutathione disulfide had no effect. Neither glutathione nor glutathione disulfide had any effect on 12α-hydroxylase activity in the absence of the microsomal stimulator (Table 2). The 12α-hydroxylase stimulatory fraction showed glutathione S-transferase activity when measured with 1-chloro-2,4-dinitrobenzene as substrate (18). The specific activity of the preparation was 5.3 μmol of product formed per mg of protein and min.

Table 3 shows that the microsomal 12α-hydroxylase stimulatory fraction also stimulated cholesterol 7α-hydroxylase activity in cytochrome P-450 LM_4.

Cytosolic 12α-hydroxylase inhibitor. A cytosolic protein fraction which had an inhibitory effect on reconstituted 12α-hydroxylase activity was obtained after polyethylene glycol fractionation and DEAE-cellulose chromatography. The inhibitory activity was eluted from the DEAE-cellulose column with a KCl-gradient. Fig. 2 shows that the inhibitory activity was dependent on the amount of protein. Maximal inhibition was obtained with 0.2 mg of protein. The protein fraction showed upon SDS-polyacrylamide gel electrophoresis a number of bands. The 12α-hydroxylase inhibitor was not dependent on ATP and $MgCl_2$ and was not affected by NaF (Table 1) or reduced glutathione or glutathione disulfide (Table 2). The fraction did not show glutathione S-transferase activity. The inhibitory activity was stable to heat treatment at 80°C for 5 min.

TABLE 3. Effect of 12α-hydroxylase modulating protein fractions on cholesterol 7α-hydroxylase activity.

System	Cholesterol 7α-hydroxylation
	pmol/nmol cytochrome P-450 x min
Control	20
Control + 12α-hydroxylase stimulator	45
Control + 12α-hydroxylase inhibitor	35

The control system contained 0.25 nmol of cytochrome P-450 LM_4, 1.5 units of NADPH-cytochrome P-450 reductase, 15 μg of dilauroylglycero-3-phosphorylcholine, 0.9 μmol of NADPH, 0.3 mg of Triton X-100, 5 mM dithiotreitiol and 25 nmol of cholesterol in a total volume of 0.75 ml of 50 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol. Incubations were performed in 37°C for 20 min. In experiments on the effects of 12α-hydroxylase stimulatory protein (2μg) and 12α-hydroxylase inhibitory protein (0.2 mg) these fractions were included in the incubation mixture.

Table 3 shows that the cytosolic 12α-hydroxylase inhibitory fraction did not inhibit cholesterol 7α-hydroxylase activity. Rather it had a slight stimulatory effect.

DISCUSSION

The results of the present investigation show that the activity of a reconstituted 12α-hydroxylase system from rabbit liver microsomes can be modulated by protein fractions from the liver cell. The 12α-hydroxylase under study was present in an appa-

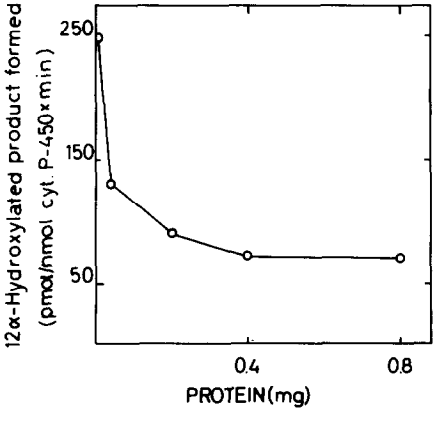

Fig. 2. Effect of cytosolic protein fraction on inhibition of reconstituted 12α-hydroxylase activity. Incubation conditions were as in Table 1 except for the varying concentrations of cytosolic protein fraction.

rently homogeneous cytochrome P-450 LM_4 fraction (10). The fact that the heat-labile stimulatory activity was highest in preparations obtained after the second sonication indicates that the 12α-hydroxylase stimulator is a microsomal protein. The stimulation was not due to the presence of cytochrome P-450, cytochrome b_5 or NADPH-cytochrome P-450 reductase in the microsomal protein fraction. When the same preparation procedures were applied to cytosol, no fractions with stimulatory effect on 12α-hydroxylase activity were obtained. Thus, the present 12α-hydroxylase stimulator differs from the cytosolic cholesterol 7α-hydroxylase stimulatory fraction which has been isolated previously in this laboratory from rat (6) and rabbit liver cytosol (19). It should be noted, however, that the microsomal 12α-hydroxylase stimulator also stimulated cholesterol 7α- hydroxylation catalyzed by cytochrome P-450 LM_4. This finding requires further study. It should be mentioned that the cholesterol 7α-hydroxylase stimulator from rat liver cytosol has been recently purified to apparent homogeneity and shown to require reduced glutathione for stimulatory activity (Danielsson, Kalles and Wikvall, submitted for publication). The lability of the present 12α-hydroxylase stimulator to storage and the reactivation of the fraction with glutathione suggest that also the 12α-hydroxylase stimulatory activity is dependent on glutathione. However, the regulation of purified cholesterol 7α-hydroxylase involves sulfhydryl groups in the cytochrome P-450. In contrast, the present results show that purified 12α-hydroxylase is not affected by glutathione and glutathione disulfide. Thus, the results indicate that the 12α-hydroxylase stimulatory protein itself is dependent on glutathione and/or reduced sulfhydryl groups for activity.

The cytosolic 12α-hydroxylase inhibitor was stable to mild heat treatment. However, the activity was retained after dialysis and the fractionation and chromatographic properties of the fraction strongly indicate that the effect is mediated by protein. The 12α-hydroxylase inhibitor did not inhibit cholesterol 7α-hydroxylase activity. It rather had a slight stimulatory effect on this activity. The differing effects of the cytosolic protein fraction on 12α-hydroxylation and cholesterol 7α-hydroxylation should be viewed in the light of the fact that the two hydroxylations are catalyzed be separate isozymes in the cytochrome P-450 LM_4 fraction (20).

The results of the present as well as of a previous communication from this laboratory (6) clearly demonstrate that modulating proteins are involved in the regulation of purified 12α-hydroxylase and cholesterol 7α-hydroxylase systems. It is also apparent that the mode of modulation of these two important hydroxylase activities in bile acid biosynthesis is not identical.

ACKNOWLEDGMENTS

The skilful technical assistance of Miss Åsa Byström and Mrs Kerstin Rönnquist is gratefully acknowledged. This work was supported by the Swedish Medical Council (Project 03X-218).

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