Regulation of bile-acid synthesis

Role of sterol carrier protein₂ in the biosynthesis of 7 α -hydroxycholesterol

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Sterol carrier protein₂ (SCP₂) is known to stimulate utilization of cholesterol in enzymic reactions in which cholesterol is the substrate. Substantial recent experimental evidence indicates that SCP₂: (i) activates enzymic conversion of intermediates between lanosterol and cholesterol; (ii) stimulates the microsomal conversion of cholesterol into cholesterol ester in rat liver; and (iii) enhances mitochondrial utilization of cholesterol for pregnenolone formation in the adrenals. The conversion of cholesterol into 7 α -hydroxycholesterol is the rate-limiting step in bile-acid synthesis. We therefore investigated the effect of SCP₂ on this physiologically critical reaction by using a gas-chromatography-mass-spectrometry procedure that measures the mass of 7 α -hydroxycholesterol formed. The results show that SCP₂ enhances 7 α -hydroxycholesterol formation by rat liver microsomes (microsomal fractions), utilizing either endogenous membrane cholesterol, cholesterol supplied exogenously in serum or in the form of cholesterol/phospholipid liposomes. Microsomes immunotitrated with anti-SCP₂ antibody exhibited considerably less capacity to synthesize 7 α -hydroxycholesterol, which was restored to control levels on addition of purified SCP₂. These data are consistent with the suggestion that SCP₂ may be of physiological significance in the overall metabolism of cholesterol.

Soluble cytosolic proteins have been described that affect cholesterol hydroxylation, although the physiological significance of their role is obscure. Thus Mitton *et al.* (1971) have reported a stimulating effect of 100000g rat liver supernatant on microsomal 7 α -hydroxycholesterol biosynthesis. On the other hand, Shefer *et al.* (1968) have found this effect to be negative. Spence & Gaylor (1977) have observed that a partially fractionated cytosolic material had the ability to stimulate cholesterol hydroxylation.

Scallen *et al.* (1974) and Johnson & Shah (1974) demonstrated the presence of two proteins in the rat liver soluble fraction that were required for the microsomal conversion of squalene into cholesterol. In later studies, Srikantaiah *et al.* (1976) purified the first of these two proteins, sterol

carrier protein₁ (SCP₁), and showed its requirement in the enzymic conversion of squalene into lanosterol by rat liver microsomal membranes. SCP₁ is identical with the supernatant protein factor purified by Ferguson & Bloch (1977). The second of the two proteins, termed 'sterol carrier protein₂', was recently purified by Noland *et al.* (1980), and it was shown to activate enzymic conversions of intermediates between lanosterol and cholesterol by liver microsomal membranes. Further studies on the role of SCP₂ in reactions that utilize cholesterol as substrate have revealed that: (i) it stimulates utilization of cholesterol, both endogenous and exogenous, in the microsomal conversion of cholesterol into cholesterol ester catalysed by acyl-CoA: cholesterol acyltransferase (Gavey *et al.*, 1981); and (ii) it stimulates transfer of cholesterol from adrenal lipid droplets to mitochondria and mitochondrial utilization of cholesterol for pregnenolone formation (Chanderbhan *et al.*, 1982).

Abbreviation used: SCP, sterol carrier protein.

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The most recent findings of experiments with SCP₂, using either a reconstituted adrenal or rat liver system, show that all cholesterol transport and activation of the conversion of 7-dehydrocholesterol into cholesterol is abolished by immunotitration of the respective cytosols with anti-SCP₂ IgG (Vahouny *et al.*, 1983).

In the present study we provide evidence (i) that a pure, homogeneous preparation of SCP₂ stimulates microsomal 7 α -hydroxycholesterol biosynthesis, and (ii) that SCP₂ is effective in promoting cholesterol hydroxylation from both endogenous microsomal cholesterol and cholesterol supplied exogenously in the form of whole serum or as cholesterol/phospholipid liposomes.

Experimental procedures

Materials

Antisera to SCP₂ were prepared in rabbits and characterized by immunoelectrophoresis (Vahouny *et al.*, 1983). Homogenous SCP₂ was prepared as described previously (Noland *et al.*, 1980). Bovine serum albumin (fraction IV, essentially fatty-acid-free) was from Sigma. Asolectin was purchased from Associated Concentrates, Woodside, NY, U.S.A. Sprague-Dawley male rats (150–220 g) used in these studies were kept in windowless quarters and under a strict 12 h reversed light/dark cycle (15.00–03.00 h light). Purina Rat Chow and water were allowed *ad libitum*. All chemicals were of highest purity grade available and were obtained from Fisher.

Liposomes

Cholesterol/phospholipid liposomes were prepared as described by Feo *et al.* (1978) and Radomska-Pyrek *et al.* (1978). A 9:1 (w/w) mixture of asolectin/cholesterol was prepared in chloroform. The solvent was evaporated under N₂ and 1.0 ml of water per 30 mg of lipid was added. The mixture was vortex-mixed at 42°C for 15 min under N₂, sonicated (Sonifer; setting 6) for 30 min in ice and was centrifuged at 134 000 g to remove unincorporated components, which sedimented in the form of a pellet. For some experiments, liposomes were further purified by column chromatography on Sepharose 4B, from which they were eluted as a single peak. Lipid phosphorus of asolectin and of liposomes was measured by the method of Bartlett (1959).

7 α -Hydroxycholesterol

Rat liver microsomes (microsomal fractions) were prepared as described previously (Sanghvi *et al.*, 1981). Microsomes with and without SCP₂ were preincubated in phosphate buffer in the absence of cofactors, as described in legends to the

Figures and Tables. Incubation, after the addition of cofactors, was for various periods as noted in Tables and Figures. 7 α -Hydroxycholesterol production was measured by a g.c.-m.s. method (Hewlett-Packard 5986A mass spectrometer) under conditions described previously (Sanghvi *et al.*, 1978, 1981). Enzyme activity was determined during the period when 7 α -hydroxycholesterol production is a linear function of both the time of incubation, as well as the microsomal protein concentration. In some experiments, only the final amount of 7 α -hydroxycholesterol formed at the end of a 60 or 90 min incubation was measured when the reaction is well past the linear phase. The acetone-dried powder of microsomes was prepared as described by Scallen *et al.* (1968). Microsomal protein was determined by the method of Bradford (1976). The microsomal protein in different assays varied between 0.3 and 0.5 mg. The total volume of each incubation flask was 700 μ l.

Each experiment was performed utilizing combined microsomes obtained from the livers of at least four rats. All assays were performed at least in duplicate. The analytical variation in the measurement of 7 α -hydroxycholesterol was less than 5%.

Results and discussion

Previous reports (Mitton *et al.*, 1971; Spence & Gaylor, 1977) have demonstrated the ability of 100 000 g rat liver supernatant, either intact or fractionated, to stimulate microsomal hydroxylation of cholesterol. SCP₂, isolated from rat liver cytosol and purified to homogeneity (Noland *et al.*, 1980), is shown to stimulate cholesterol transport from adrenal lipid droplets to mitochondria and to stimulate the utilization of cholesterol in enzymic reactions in which cholesterol is the substrate (Gavey *et al.*, 1981). This information provided the basis for the present work on the role of SCP₂ in microsomal hydroxylation of cholesterol. In preliminary experiments, conducted in collaboration with the laboratory of Dr. G. S. Boyd (now deceased), University of Edinburgh Medical School, a concentration-dependent increase in the microsomal conversion of [4-¹⁴C]cholesterol to labelled 7 α -hydroxycholesterol by SCP₂ was observed (B. J. Noland, I. F. Craig, M. E. Lawson, T. J. Scallen & G. S. Boyd, unpublished work). Confirmation of these previous observations by more detailed experiments is reported here.

In order to observe an SCP₂ effect on 7 α -hydroxylase, we found that it was necessary to preincubate SCP₂ with microsomes in the absence of cofactors. The greatest increase in SCP₂-stimulated 7 α -hydroxycholesterol production occurs in the first 30 min of preincubation, with little further increase up to 180 min (Fig. 1). Therefore

Fig. 1. Effect of preincubation of SCP₂ and microsomes on 7 α -hydroxycholesterol formation

Microsomes with (○) and without (●) SCP₂ were preincubated at 37°C in the absence of cofactors for various times. The final amount of 7 α -hydroxycholesterol formed at the end of a 60 min incubation with cofactors was then measured.

all subsequent experiments were performed with a 30 or 60 min preincubation time. The effects of SCP₂ were studied during both the early, linear phase of the reaction ('enzyme activity') and during the late, stable, phase of the reaction ('final amount formed').

Stimulation of microsomal cholesterol 7 α -hydroxylase activity by SCP₂ is shown in Fig. 2, where the presence of SCP₂ in the reaction almost doubles the enzyme activity from 63 to 103 pmol/min per mg of protein. When rat liver microsomes were incubated for 90 min in the presence of SCP₂, there was a significant increase in the final amount of 7 α -hydroxycholesterol produced. If similar incubations were carried out in the presence of increasing amounts of SCP₂, there was, at first, a linear increase in the final amount of 7 α -hydroxycholesterol produced. When the SCP₂ concentration was raised above 4 μ M, this increase was no longer linear and in fact, stabilized at an SCP₂ concentration of 8 μ M. Thus the data in Fig. 3 give the appearance of a saturation plot. In Fig. 4 the amount of SCP₂ in the incubations is expressed in relation to the amount of microsomal protein and, again, the increase in the production of hydroxylated sterol is proportional to the amount of SCP₂. These data collectively show that SCP₂ was capable of accelerating 7 α -hydroxylase activity and increasing the final amount of hydroxycholesterol formed, when endogenous microsomal cholesterol was utilized.

The next series of experiments was carried out to see whether SCP₂ is similarly effective when cholesterol is supplied by an exogenous source, such as cholesterol-containing phospholipid liposomes. As shown in Fig. 5, phospholipid/choles-

Fig. 2. Stimulation of cholesterol 7 α -hydroxylase activity by SCP₂

Microsomes were preincubated with (●) and without SCP₂ (○) at 37°C for 60 min. Cofactors were then added and enzyme activity was measured during the linear phase.

Fig. 3. Enhanced 7 α -hydroxycholesterol production with increasing amounts of SCP₂

Microsomes were preincubated with SCP₂ (0–8 μ M) at 37°C for 60 min. 7 α -Hydroxycholesterol formation was then monitored at the end of 90 min incubation with cofactors.

terol liposomes alone bring about an increase in both microsomal 7 α -hydroxylase activity and the final amount of hydroxycholesterol formed. The addition of SCP₂ under these conditions has no effect on enzyme activity, but it does markedly increase the final amount of hydroxylated sterol produced at the end of 90 min incubation. Moreover, the results in Table 1 show that when phospholipid/cholesterol liposomes serve as an additional source of substrate cholesterol, the increase in the final amount of 7 α -hydroxycholesterol formed depends on the amount of SCP₂.

Fig. 4. Enhanced 7 α -hydroxycholesterol production as a function of the SCP₂/protein ratio

Microsomes were preincubated with SCP₂ (0–8 μ M) at 37°C for 60 min. 7 α -Hydroxycholesterol formation was then monitored at the end of 90 min incubation with cofactors. The percentage increase compared with the control is presented.

Fig. 5. Effect of SCP₂ and liposomes on 7 α -hydroxycholesterol formation

After a 60 min preincubation of microsomes (●) at 37°C with SCP₂ (1.5 μ M) and asolectin/cholesterol liposomes (□) or with liposomes alone (△), the 7 α -hydroxycholesterol formation at indicated times was monitored. The basal levels of 7 α -hydroxycholesterol in boiled controls of respective preparations are subtracted from all values.

Recently we have demonstrated (Sanghvi *et al.*, 1984) that an acetone-dried-powder preparation of rat liver microsomes, which is completely devoid of non-esterified cholesterol, exhibits no 7 α -hydroxylase activity unless an exogenous source of substrate cholesterol is provided. Results in Table 2 indicate that SCP₂ brings about an increase in acetone-dried-powder 7 α -hydroxylase activity, when either liposomes or serum is the donor of substrate cholesterol. Again, the change in enzyme

Table 1. Effect of different concentrations of SCP₂ on 7 α -hydroxycholesterol production in microsomes with and without liposomes

Microsomes with and without liposomes and/or SCP₂ were preincubated at 37°C for 30 min before the addition of cofactors. 7 α -Hydroxycholesterol formed at the end of 90 min of incubation with cofactors was measured. The cholesterol content of the liposomes was 40 μ g.

	7 α -Hydroxycholesterol (pmol/mg of protein)	
	– Liposomes	+ Liposomes
Microsomes	1733	2400
+ SCP ₂ (2 μ M)	2244	2800
+ SCP ₂ (4 μ M)	2800	3180

Table 2. Effect of SCP₂ on cholesterol 7 α -hydroxylase activity in microsomal acetone-dried powder

Microsomal acetone-dried powder was preincubated at 37°C with liposomes or serum without cofactors for 30 min. After the addition of cofactors, cholesterol 7 α -hydroxylase activity was measured over a period of 10 min during the linear phase. Serum and liposomes contained 15 and 12 μ g respectively of non-esterified cholesterol.

Experiment	Cholesterol 7 α -hydroxylase activity (pmol/min per mg of protein)
(a) Microsome powder	
+ liposomes	10
+ SCP ₂ (5 μ g)	18
(b) Microsome powder	
+ serum	12
+ SCP ₂ (23 μ g)	22
+ SCP ₂ (46 μ g)	32

activity is proportional to the amount of SCP₂ added. Thus SCP₂ behaves similarly towards both the acetone-dried powder and intact microsomes.

The specificity of SCP₂ in stimulating cholesterol 7 α -hydroxylase was assessed in an experiment in which we compared its effects to that of albumin and fatty-acid-binding protein. The results in Fig. 6 show that, whereas SCP₂ increases 7 α -hydroxycholesterol production, equimolar albumin and fatty-acid-binding protein are without effect.

Since SCP₂ is derived from rat liver cytosol, we reasoned that treatment of rat liver cytosol with anti-SCP₂ IgG should compromise the 7 α -hydroxylase-stimulating ability of cytosol. In a number of experiments carried out to investigate this phenomenon, we observed that immunotitration of cytosol by anti-SCP₂ IgG did not produce a significant decrease in the ability of cytosol to

enhance 7α-hydroxycholesterol synthesis. In an attempt to confirm this finding, we treated rat liver cytosol with anti-SCP₂ IgG and then tested its ability to affect adrenal mitochondrial pregnenolone production. Results in Table 3(a) show that cytosol treated with anti-SCP₂ has indeed lost its ability to enhance pregnenolone production by adrenal mitochondria, whereas the native cytosol, or cytosol treated with non-immune IgG, retains this ability. These same fractions were then tested for their effect on 7α-hydroxycholesterol synthesis. Again, consistent with our previous observations, the anti-SCP₂-treated cytosol showed virtually the same ability to stimulate 7α-hydroxycholesterol formation as did the cytosol treated with non-immune IgG (Table 3b). These results suggest that rat liver cytosol contains protein(s) or non-protein components besides SCP₂ with the ability to enhance 7α-hydroxylation of cholesterol. This observation is consistent with reports suggesting the presence in rat liver cytosol of such factors (Craig *et al.*, 1979; Danielsson *et al.*, 1980, 1984).

Fig. 6. Specificity of SCP₂ in stimulating microsomal 7α-hydroxycholesterol synthesis

Microsomes were preincubated at 37°C for 60 min in the absence of cofactors with SCP₂ (2 μM), albumin (ALB, 4 μM), and fatty-acid-binding protein (FABP, 2 μM). The final amount of 7α-hydroxycholesterol formed at the end of a 90 min incubation with cofactors was then measured. The data are means ± S.D., n = 4. The significance values are with respect to control (N.S., not significant).

The presence of endogenous SCP₂ in microsomes was ascertained by direct titration of microsomes with anti-SCP₂ IgG. Fig. 7 shows that immunotitrated microsomes form progressively less 7α-hydroxycholesterol relative to microsomes treated with non-immune IgG or untreated microsomes. Importantly, when SCP₂ is added to immunotitrated microsomes they are able to form 7α-hydroxycholesterol at levels comparable with those in microsomes treated with non-immune IgG (Table 4). This series of experiments would seem to

Fig. 7. Immunotitration of microsomal 7α-hydroxycholesterol synthesis by anti-SCP₂ IgG

Indicated ratios of microsomes and anti-SCP₂ IgG were incubated at 25°C for 1 h. They were then left overnight at 4°C. The tubes were shaken very gently and centrifuged at 2200 rev./min. (Beckman Model TJ-6 refrigerated centrifuge) for 30 min. The supernatant was decanted, leaving behind a pellet. Microsomes, recovered after centrifugation of supernatant for 20 min at 300000g, were preincubated for 60 min at 37°C and 7α-hydroxycholesterol formation was measured at the end of a 90 min incubation with cofactors. The control 7α-hydroxycholesterol levels in untreated microsomes, or in microsomes treated with nonimmune IgG, were 2653 and 2651 pmol/mg of protein respectively.

Table 3. Effect of anti-SCP₂-treated liver cytosol on (b) 7α-hydroxycholesterol and (a) pregnenolone synthesis
Microsomes with and without additions were preincubated at 37°C for 30 min. 7α-Hydroxycholesterol produced at the end of a 90 min incubation with cofactors was then measured (b).

Expt.	(a) Pregnenolone (ng/mg of adrenal mitochondrial protein)	(b) 7α-Hydroxycholesterol (pmol/mg of microsomal protein)
Control	50.4	548
+ Cytosol	153.0	2311
+ Cytosol + IgG _{non-immune}	146.0	1932
+ Cytosol + IgG _{immune}	53.0	1830

Table 4. *Effect of SCP₂ on 7 α -hydroxycholesterol synthesis in microsomes treated with anti-SCP₂ IgG*
Conditions for the experiments were the same as those in Fig. 7.

Expt.	7 α -Hydroxycholesterol produced (pmol/mg of protein)
(a) Microsomes (M)	1739
+ Non-immune IgG (M-IgG _c)	1499
+ Immune IgG (M-IgG _i)	930
(b) M-IgG _i + SCP ₂ (8 μ M)	1444

confirm the presence of SCP₂ in microsomes and indicate a possible role for it in 7 α -hydroxycholesterol biosynthesis.

In all our experiments we have noticed that the magnitude of stimulation of 7 α -hydroxylation of cholesterol brought about by SCP₂ is typically a doubling, relative to controls. This is in contrast with changes of much higher magnitude observed by Gavey *et al.* (1981) with respect to the stimulation of cholesterol ester formation and by Chanderbhan *et al.* (1982) with respect to stimulation of transfer of cholesterol from adrenal lipid droplets to mitochondria and mitochondrial utilization of cholesterol for pregnenolone formation by SCP₂. It is not clear, on the basis of available data, why such a difference should exist. It may be that microsomes have greater amounts of SCP₂ than mitochondria and may, therefore, have been prestimulated with respect to 7 α -hydroxylase. At present, it is not possible to measure directly the endogeneous levels of SCP₂. Secondly, it is also likely that SCP₂-sensitive enzymes are differentially affected by SCP₂. This possibility may be explored in experiments with highly purified enzyme preparations. Unfortunately, this again is not possible at the present time.

In summary, the results presented here demonstrate that SCP₂ can enhance 7 α -hydroxycholesterol formation by rat liver microsomes under various experimental conditions. It is important to note that SCP₂ is able to accomplish this regardless of the source of substrate cholesterol. These observations are compatible with the data of Gavey *et al.* (1981) and Chanderbhan *et al.* (1982), which show that SCP₂ promotes utilization of cholesterol by rat liver microsomes in the formation of cholesterol esters catalysed by acyl-CoA: cholesterol acyltransferase and the formation of pregnenolone by the adrenal mitochondria. In these instances there is evidence suggesting that SCP₂ serves as the specific intracellular transport system that delivers either endogenous or exogenous cholesterol to the respective enzymes that further metabolize it.

We do not as yet know the mechanism by which SCP₂ appears to promote cholesterol utilization in the formation of 7 α -hydroxycholesterol. It might function to improve the accessibility, as well as

availability, of substrate cholesterol and, in addition, play some role in the removal of the product, 7 α -hydroxycholesterol.

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