EVIDENCE FOR THE PARTICIPATION OF TWO SOLUBLE NONCATALYTIC PROTEINS IN HEPATIC MICROSOMAL CHOLESTEROL SYNTHESIS[1]

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SUMMARY

The capacity of liver soluble fraction to stimulate hepatic microsomal conversion of squalene to cholesterol is lost on treatment with trypsin. Heat treatment of the soluble fraction results in a selective loss of its capacity to stimulate conversion of squalene to cholesterol; the ability to stimulate conversion of lanosterol and desmosterol to cholesterol is however retained. It is proposed that the liver soluble fraction contains at least two noncatalytic proteins, one heat-labile and the other heat-stable, which participate in microsomal cholesterol synthesis. The heat-labile protein mediates the conversion of squalene to lanosterol while the heat-stable protein is needed for the conversion of lanosterol and other sterol precursors to cholesterol.

A requirement for a noncatalytic soluble protein for hepatic microsomal synthesis of cholesterol from squalene and from other sterol precursors, has been clearly demonstrated (1-10). The squalene and sterol carrier protein (SCP) which binds squalene, sterols and phospholipids and stimulates microsomal sterol synthesis has been purified in the laboratories of two groups of investigators (4-9). The major discrepancy in the results of these studies however, is that the protein purified by Ritter and Dempsey (4-6) is heat-stable while the protein purified by Scallen's group (7-9) is heat-labile. Recently, Tai and Bloch (10) have purified a liver supernatant protein factor (SPF) which does not bind squalene or oxidosqualene, but stimulates microsomal squalene epoxidase and is heat-labile. To resolve these differences, we have reexamined, in the present study, the effects of heat treatment and of trypsin digestion of the 105,000 g supernatant fraction from liver on its capacity to stimulate 1) the

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overall conversion of squalene to cholesterol, 2) the conversion of lanosterol to cholesterol, and 3) the reduction of desmosterol to cholesterol. The results obtained support the hypothesis that the liver soluble fraction contains two protein factors, one heat-labile and the other heat-stable. The protein which is heat-labile, is required for squalene epoxidase system and the protein which is heat-stable participates in microsomal cholesterol synthesis beyond the lanosterol step.

Materials and Methods

[^{14}C] Squalene and [^{14}C] lanosterol were prepared from DL-2 [^{14}C] mevalonic acid DBED salt (New England Nuclear Corp., Boston, Ma.) by the method of Tchen (11). 26 [^{14}C] Desmosterol was purchased from New England Nuclear Corp., Boston, Ma., NADPH, NAD , glucose-6-phosphate and soybean trypsin inhibitor were purchased from Calbiochem, San Diego, Ca. Glucose-6-phosphate dehydrogenase and trypsin, were purchased from Sigma Chemical Co., St. Louis, Mo. Precoated silica gel G plates and silver nitrate were purchased from Eastman-Kodak, Rochester, N. Y.

Adult female Sprague-Dawley rats weighing approximately 200 gm were sacrificed by decapitation. The livers were removed, perfused with saline and homogenized in 2.5 volumes of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM DTT. The homogenate was centrifuged at 1,000 g for 10 min, and the supernatant decanted and centrifuged at 18,000 g for 20 min. The 18,000 g supernatant was then centrifuged at 105,000 g for 90 min and the floating layer of lipid drawn off. The upper two-thirds portion of the supernatant fraction (S_{105}) was collected and recentrifuged at 105,000 g for 60 min to remove any residual microsomal material. The sedimented microsomal pellet (MS) was resuspended in a buffer volume equal to that of the 18,000 g supernatant and recentrifuged at 105,000 g for 60 min. The supernatant fraction and washed microsomal pellets were stored at -86° if not used immediately.

The protein fraction was obtained from S_{105} by precipitation with ammonium sulfate between 40-70% saturation. The precipitate (AS) was redissolved in a

TABLE I

CONVERSION OF SQUALENE AND STEROL PRECURSORS TO CHOLESTEROL BY RAT LIVER MICROSOMES

Substrate	Additions	Protein (mg)	% [^{14}C] Recovered in Cholesterol*	Stimulation (-fold)
[^{14}C] Squalene	None	1.5	1.0	-
	S_{105}***	7.5	1.7	-
	S_{105}	9.0	9.8	9.8
	S_{105}**	5.0	1.1	1.1
	AS(40-70%)	5.3	9.8	9.8
	AS(40-70%)**	3.6	0.9	0
[^{14}C] Lanosterol	None	1.5	1.63	-
	S_{105}***	7.5	2.05	-
	S_{105}	9.0	21.8	13.4
	S_{105}**	5.0	18.1	11.1
	AS(40-70%)	5.0	22.5	13.7
	AS(40-70%)**	3.8	17.1	10.5
[^{14}C] Desmosterol	None	1.5	4.7	-
	S_{105}***	7.5	3.6	-
	S_{105}	9.0	52.5	11.2
	S_{105}**	5.0	50.3	10.7
	AS(40-70%)	5.3	53.8	11.9
	AS(40-70%)**	3.6	51.7	11.0

Each reaction mixture contained 1.5 mg washed microsomal protein, 100 mM phosphate buffer, pH 7.4 and 1.2 mM NADPH. Squalene experiments contained in addition, 0.6 mM NAD^+, 30 mM nicotinamide, 4.0 mM glucose-6-PO_4 and 1 unit glucose-6-PO_4 dehydrogenase. Labeled substrates (20,000 cpm) in 5 µl dioxane:propylene glycol (2:1) were routinely preincubated with S_{105} or AS preparations at 37° for 15 min under N_2. Reactions were initiated by the addition of microsomes, and incubations were carried out aerobically (squalene and lanosterol substrates) for a period of 120 min or anaerobically (desmosterol substrate) for a period of 60 min. Protein was estimated by biuret method (12). Reaction products were isolated, separated by thin layer chromatography and assayed for radioactivity as previously described (13, 14).

* Values are the mean of closely agreeing duplicates
** Heated at 100° for 1 min
*** Microsomes omitted

TABLE II

EFFECT OF TRYPSIN HYDROLYSIS ON THE CAPABILITY OF THE SOLUBLE PROTEIN FRACTION PRECIPITATED BY AMMONIUM SULFATE TO STIMULATE MICROSOMAL CHOLESTEROL SYNTHESIS

Substrate	Additions	Protein (mg)	% [^{14}C] Recovered in Cholesterol*	Stimulation (-fold)
[^{14}C] Squalene	None	1.5	0.92	-
	AS	5.3	9.6	10.4
	AS + Tryp	3.5	1.8	1.9
	AS + Tryp + Tryp Inhbtr.	5.1	9.0	9.8
[^{14}C] Lanosterol	None	1.5	1.6	-
	AS**	4.0	17.1	10.7
	AS** + Tryp	2.7	4.6	2.8
	AS** + Tryp + Tryp Inhbtr.	3.7	15.4	9.7
[^{14}C] Desmosterol	None	1.5	4.3	-
	AS**	3.7	49.9	11.6
	AS** + Tryp	2.8	7.1	1.7
	AS** + Tryp + Tryp Inhbtr.	3.6	46.4	10.8

AS (40-70%, 8.0 mg) in 100 mM phosphate buffer, pH 8.5, were digested with 0.8 mg of trypsin for 30 min at 37°. Proteolysis was stopped by the addition of equivalent amounts of soybean trypsin inhibitor and incubating the digests an additional 15 min. Control flasks containing 40 mg AS (40-70%), to which 0.8 mg each of trypsin and soybean trypsin inhibitor were simultaneously added, were incubated for 45 min. After the incubations the pH was adjusted to 7.4. Aliquots of the trypsin treated and control samples were then tested for its ability to stimulate conversion of squalene, lanosterol and desmosterol to cholesterol by liver microsomes as described in Table I.

* Values are the means of closely agreeing duplicate determinations
** Heated at 100° for 1 min

volume of buffer equivalent to that of the original S_{105} fraction, dialyzed against 100 volumes of the same buffer for 12 hr, and assayed for its ability to stimulate conversions of squalene, lanosterol and desmosterol to cholesterol. To test the heat stability of AS, the fractions containing 5% $(NH_4)_2SO_4$, w/v, were heated under nitrogen at 100° for one min. The coagulated protein was sedimented and the supernatant fluids assayed for their ability to stimulate squalene, lanosterol or desmosterol conversion to cholesterol.

RESULTS

The conversion of squalene, lanosterol and desmosterol to cholesterol by rat liver microsomes is shown in Table I. The presence of the supernatant fraction stimulated the conversion of squalene to cholesterol 10-fold, lanosterol to cholesterol 13-fold, and desmosterol to cholesterol 11-fold. The heat treatment of the supernatant fraction resulted in a loss of its capacity to stimulate the conversion of squalene to cholesterol; however, no loss in the capacity to stimulate the conversion of lanosterol and desmosterol to cholesterol by liver microsomes was observed. The studies with the protein fraction from liver supernatant precipitated with ammonium sulfate at 40-70% saturation, which has been used by other investigators, as the source for the purification of the SCP and SPF proteins gave essentially similar results. Treatment with trypsin of the unheated protein fraction precipitated by ammonium sulfate results in a substantial loss of its activator capacity for the conversion of squalene to cholesterol by microsomes (Table II). Similarly, treatment with trypsin of heated ammonium sulfate fraction results in an almost complete loss of its capacity to stimulate conversion of lanosterol and desmosterol to cholesterol.

DISCUSSION

The data presented here show that the capacity of the soluble fraction to stimulate conversion of squalene to cholesterol is lost by trypsin treatment and by heating at 100° for one minute. However, the capacity of the soluble fraction to stimulate the conversion of lanosterol and desmosterol is unaffected by heat treatment, but is lost by trypsin treatment of the heated fraction. The results therefore support the hypothesis that at least two soluble proteins participate in the overall conversion of squalene to cholesterol by liver microsomes. They further indicate that the conversion of lanosterol or sterol precursors to cholesterol requires a heat-stable protein factor while the overall conversion of squalene to cholesterol requires both heat-labile and heat-stable proteins. It can be inferred therefore that the heat-labile protein factor plays

a role in the conversion of squalene to lanosterol. The results of Yamamoto, Lin and Bloch (15), that microsomal 2, 3, oxidosqualene cyclase does not require the supernatant factor, are in accord with the suggestion that the heat-labile protein is required solely for the squalene epoxidase system.

The results of the previous investigators can be reconciled in the following manner. The heat-labile protein which is required for conversion of squalene to lanosterol is probably identical with the supernatant protein factor (SPF) purified by Tai and Bloch (10) and which participates in the squalene epoxidase system. The heat-stable protein which stimulates the conversion of lanosterol and desmosterol to cholesterol by liver microsomes is perhaps identical with that reported by Ritter and Dempsey (4-6). While the binding of squalene to this protein has been reported (6) its ability to stimulate the enzymatic steps between squalene and lanosterol has not been adequately documented.

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