CHARACTERIZATION OF NATIVE STEROL CARRIER PROTEIN

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1. Introduction

Recently we made the proposal and presented evidence that a noncatalytic carrier protein (*sterol carrier protein* or SCP) is involved in the conversion of squalene into cholesterol by liver microsomes [1–7]. This was based on observations made with an acetone powder of rat liver microsomes which required the 105,000 *g* supernatant (S_{105}) of rat liver for this conversion [8, 9].

We now report the following accomplishments: a) the purification of native SCP from unheated S_{105} of rat liver; b) when purified SCP is added to an inactive buffer-washed microsomal preparation from rat liver, it is capable of reconstituting the entire biosynthetic sequence from squalene to cholesterol; c) SCP binds not only water-insoluble precursors of cholesterol, but also lipid components of lipoprotein, i.e., cholesterol, cholesterol ester, phospholipid and tryglyceride; d) the amino acid composition of native SCP closely resembles the amino acid composition of the protein moiety of serum low density lipoprotein (LDL). These findings are compatible with the novel hypothesis that SCP simultaneously serves both as a carrier for the enzymatic synthesis of cholesterol and as the protein component of LDL, specifying LDL assembly. Thus the enzymatic synthesis by the liver of lipid components (e.g., cholesterol) of LDL may coincide with LDL assembly.

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2. Materials and methods

2.1. *Substrates and materials*

Radioactive substrates were purchased from New England Nuclear or prepared biosynthetically for [^{3}H]squalene [8], 4,4-dimethyl-Δ^8-cholesten-3β-ol and 4β-methyl-Δ^8-cholesten-3β-ol [10]. Cγ alumina gel was obtained from Sigma; cellulose (Whatman CF-11) from Reeve Angel.

2.2. *Purification of sterol carrier protein (SCP)*

The 105,000 *g* supernatant (S_{105}) of rat liver was perpared as previously described [8] using male Sprague-Dawley rats. All buffers employed during purification of SCP contained 1×10^{-4} M EDTA and 1×10^{-3} M reduced glutathione; the pH was maintained at 7.4.

The first step in the purification of SCP from unheated S_{105} was precipitation with buffered ammonium sulfate between 50–70% saturation; the precipitate was redissolved in 0.02 M potassium phosphate buffer and dialyzed against the same buffer. This solution was then applied to a Cγ alumina gel-cellulose column (3 g alumina gel to 60 g of cellulose, 4 × 25 cm). The column was eluted with 0.02 M phosphate buffer until no more protein was eluted (1200 ml). This fraction was discarded. Next a gradient (0.02–0.15 M phosphate buffer) was used for elution. SCP activity was associated with the first major protein peak eluted by the gradient. Since resolution from a second contaminating protein peak was incomplete, the process was repeated on a second Cγ alumina gel-cellulose column (1 g alumina gel: 60 g cellulose, 4 × 25 cm) with elution by a phosphate buffer gra-

dient (0.02–0.10 M). The protein in the fractions was pooled, concentrated, dialyzed, and applied to a Sephadex G-200 column (2 × 70 cm). In some cases the protein was concentrated by precipitation with buffered ammonium sulfate, 55–65% saturation, before application to Sephadex G-200. The column was eluted with a 0.02 M phosphate buffer. The first protein peak (elution volume approx. 150 ml) was discarded; the second protein peak (elution volume approx. 184 ml) contained SCP. These fractions were pooled, concentrated, and frozen. SCP is stable when frozen for a period of up to about 2 weeks, but it loses activity when stored for longer periods of time.

The above purification scheme when applied to 9.5 g of protein (400 ml S_{105}) yielded approx. 20 mg of purified SCP.

2.3. *Incubations*

Microsomes were prepared as previously described [8] except for the addition of a washing step using 0.02 M phosphate buffer containing 1×10^{-4} M EDTA. The resulting buffer-washed microsomes were either used fresh of frozen in liquid nitrogen and stored at −80°. Incubations were conducted as previously described [3, 8] for 2 hr at 37° under oxygen. See figure legends for the contents of each flask.

Reaction products were extracted into light petroleum as previously described [8]. This extract was evaporated to dryness under a stream of nitrogen, 100 μl of benzene added, and 15 μl of this solution was applied to an Eastman Kodak silica gel sheet (1 × 20 cm). Elution was conducted with 1:2 chloroform: carbon tetrachloride. The strip was then cut into 34 segments, each 0.5 cm wide, and then each segment was placed in a scintillation vial. Toluene (10 ml) containing 0.3% diphenyloxazole was added to each vial, and the contents were shaken for 30 min. The radioactivity of each fraction was assayed by a Packard 3375 spectrometer.

2.4. *Binding studies*

One milliliter of a solution containing SCP (after

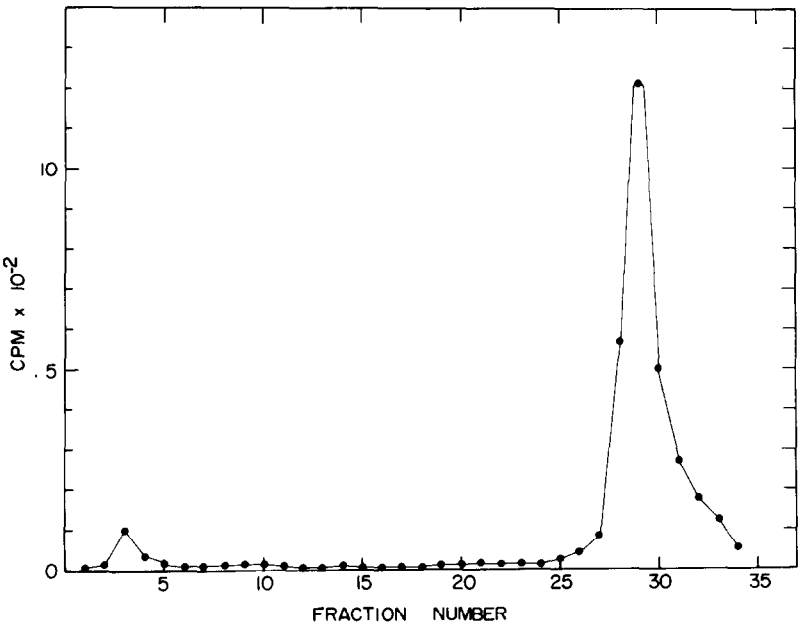

Fig. 1. Distribution of radioactivity on a silica-gel thin-layer chromatogram after a two-hour incubation of [^{3}H]squalene with a buffer-washed rat liver microsomal preparation in the absence of added SCP. The incubation flask contained (a) microsomal suspension (0.2 ml), (b) buffer (3.8 ml), (c) NADPH and NAD dissolved in buffer (1 ml) to give final concentrations of 1.2×10^{-3}M and 3×10^{-3}M, respectively, and (d) [^{3}H]squalene (44,400 cpm) added as a solution in 2:1 dioxane:propylene glycol (30 μl) [8]. The final concentration of microsomal protein was 1.20 mg/ml. An aliquot containing 3500 cpm was used for chromatography; (•–•–•) counts per minute.

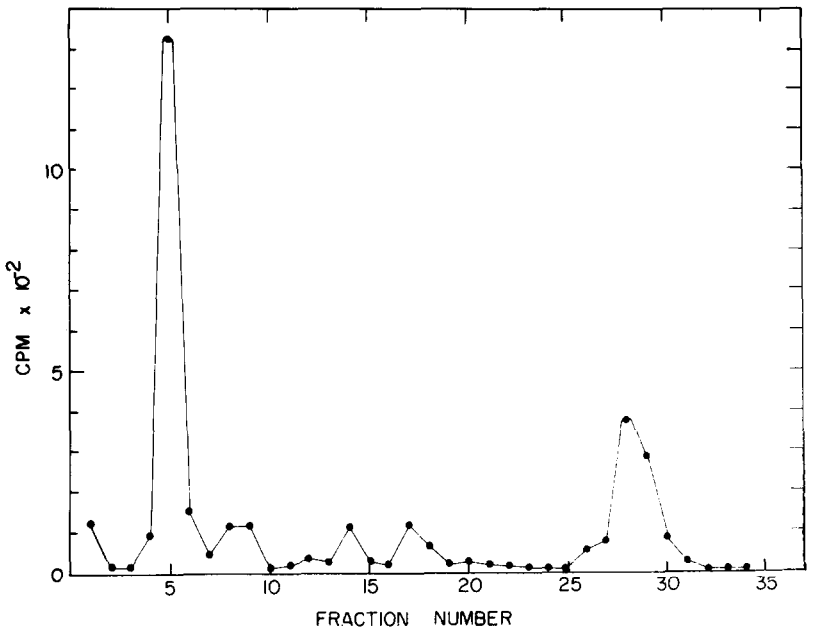

Fig. 2. Distribution of radioactivity on a silica-gel thin-layer chromatogram after a two-hour incubation of [^{3}H]squalene with a buffer-washed rat liver microsomal preparation in the presence of added purified SCP. The incubation flask contained (a) microsomal suspension (0.2 ml), (b) purified SCP (2 ml), (c) buffer (1.8 ml), (d) NADPH and NAD dissolved in buffer (1 ml) to give final concentrations of 1.2×10^{-3} M and 3×10^{-3} M, respectively, and (e) [^{3}H]squalene (44,400 cpm) added as a solution in 2:1 dioxane: propylene glycol (30 μl). The final incubation volume was 5 ml. The final conc. of microsomal protein was 1.20 mg/ml. The final conc. of SCP protein was 0.53 mg/ml. This yields a SCP protein/microsomal protein ratio 0.44. Excellent conversions of squalene into sterol (i.e., 60%) also occurred when this ratio was equal to 0.11. An aliquot containing 3500 cpm was used for chromatography; (•–•–•) counts per minute.

alumina gel purification) was used for each binding experiment. The radioactive substrate was added (in amounts of 10 nmoles or less) in 5–10 μl of 2:1 dioxane:propylene glycol [3, 8] or in buffer, depending upon solubility. Within this range the percentage of substrate bound for a given substrate remained constant. The incubation was conducted in a Dubnoff shaker for 15 min under nitrogen at 37°. An aliquot (0.05 ml) was taken for measurement of radioactivity and the remaining incubation contents were placed on a Sephadex G-25 column (2 X 25 cm); 0.02 M phosphate buffer (containing 1×10^{-4} M EDTA) was the eluting solvent, and 3 ml fractions were collected. Aliquots from each fraction were assayed for radioactivity and protein. The radioactivity associated with the protein fractions constituted the protein-bound radioactivity. This quantity was divided by the total radioactivity applied to the column to yield the percentage of radioactivity bound to protein.

2.5. *Amino acid analysis*

Purified SCP was hydrolyzed with redistilled 6 N HCl *in vaccuo* at 110° for 40 hr. The resulting amino acids were analyzed using a Spinco Model 120C amino acid analyzer.

3. Results

3.1. *Properties of purified native SCP*

Purification of SCP from unheated liver S_{105} yielded a colorless protein solution with a 280 nm/260 nm ratio of approx. 1.8. Polyacrylamide gel electropheresis at pH 7.4 yielded a single slow-moving band which just entered the separating gel. SCP sedimented as a homogeneous peak in the analytical ultracentrifuge with s = 3.80 S. Although SCP migrated on Sephadex G-200 consistent with a molecular weight of approx. 50,000, a molecular weight determined by the menis-

Table 1
Binding of lipid components of lipoprotein and other compounds to SCP.

Compound	Percentage of radioactivity bound to protein
[^{3}H]Squalene	78
[26-^{14}C]Desmosterol	59
[1α-^{3}H]Cholesterol	74
[1-^{14}C]Acetate	0.9
[2-^{3}H]Mevalonate	5
[4-^{14}C]Testosterone	0.9
[4-^{14}C]Estradiol	0.3
[1,2-^{3}H]Cortisol	21
[1-^{14}C]Triolein	78
[1,2-^{14}C]Phosphatidylcholine	84
Cholesteryl-[7-^{3}H]oleate	69
Palmityl-[1-^{14}C]CoA	99
[1-^{14}C]Oleic acid	72
[^{14}C]D-glucose	0
[2-^{14}C]Glycerol*	0
L-[^{14}C]glycerol 1-phosphate	1.1

* Binding experiments were preformed with the substrate added in either buffer or 2:1 dioxane:propyleneglycol.

Table 2
Amino acid composition of native sterol carrier protein (SCP) and the protein moiety of rat serum low density lipoprotein (LDL).

	SCP	LDL protein [14]
	(moles/10^3 moles of amino acids)	
Lys	87.7	88.5
His	16.2	19.4
Arg	50.5	54.0
Asp	105.2	100.0
Thr	56.2	58.3
Ser	69.8	77.2
Glu	116.5	130.0
Pro	59.6	37.8
Gly	65.9	49.1
Ala	67.3	62.6
Val	68.0	55.6
Ile	53.0	45.2
Leu	105.2	118.0
Tyr	28.0	26.1
Phe	49.8	45.4
Met	+	23.3
1/2 Cys	+	tr
Try	+	8.5

+: The amino acid was detected, but suitable quantitative data is not yet available; tr: Trace.

cus depletion sedimentation equilibrium method [11] gave a value of 28,000.

Lipid analysis of purified SCP by thin-layer chromatography showed only trace amounts of endogenous lipid (less than 1% of the protein mass). Lipids detected at this level induced free fatty acid, triglyceride, cholesterol and phospholipid. These results confirmed our earlier finding that SCP does not float in the ultracentrifuge at densities (1.21) which float lipoportein [12].

3.2. *Biological activity of purified SCP*

Fig. 1 shows a silicic acid chromatogram from a control experiment in which a buffer-washed rat liver microsomal preparation was incubated with [^{3}H]squalene in the absence of added SCP. The substrate [^{3}H]squalene, appears in fractions 27–34; a small product peak is seen in the 27-carbon-atom sterol region (fraction 3). Only a 7% conversion of squalene into sterols occurred in this experiment, with 3.5% of the recovered radioactivity present in the 27-carbon-atom sterol region.

Fig. 2 shows a silicic acid chromatogram of an experiment exactly the same as in fig. 1 except for the addition of purified SCP. The following points should be noted: a) When SCP is added there is a tremendous increase in the size of the 27-carbon-atom sterol peak (fractions 4–6); approx. 50% of the recovered radioactivity is associated with this peak. This represents an approx. 15-fold stimulation when compared with the control (fig. 1). In several experiments similar to the one shown in fig. 2, the amount of radioactive cholesterol in this peak has varied from 20 to 50% as demonstrated by passage through the dibromide [13]. b) Note the much smaller squalene peak (fractions 27–31) in fig. 2. c) Several small peaks which represent intermediates in the conversion of squalene into cholesterol are seen. d) The overall conversion of squalene into sterol in this experiment was 73%, compared to 7% in the absence of added SCP.

3.3. *Binding studies*

Table 1 shows the results obtained in binding experiments of SCP with several compounds. The following conclusions can be made: a) Cholesterol and its water-insoluble precursors (e.g., squalene and

desmosterol) show significant binding to SCP. We have also demonstrated binding to SCP of several other cholesterol precursors not shown in table 1, e.g., squalene-2,3-oxide-^{3}H, 4,4-dimethyl-Δ^{8} cholesten-3β-ol-^{3}H and 4β-methyl-Δ^{8}-cholesten-3β-ol-^{3}H. b) The two water-soluble precursors of cholesterol studied (acetate and mevalonate) did not show significant binding to SCP. c) There is a high specificity of SCP towards steroid structure in that [4-^{14}C]testosterone and [4-^{14}C]estradiol were not bound appreciably (less than 1% bound) and cortisol was only slightly bound. d) Lipid components of lipoprotein, e.g., triglyceride (triolein), phospholipid (phosphatidylcholine), and cholesterol ester (cholesteryl oleate) showed a high affinity for SCP. In addition, palmityl CoA and oleic acid were bound in high yield. e) Radioactive glycerol, glycerol phosphate, and glucose did not bind significantly to SCP.

3.4. *Amino acid analysis of SCP*

The amino acid composition of purified SCP is shown in table 2. For comparison the amino acid composition of the protein moiety of serum low density lipoprotein (LDL) from male Sprague-Dawley rats as determined by Koga et al. [14] is also shown. A good correlation exists between SCP and LDL protein for lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, alanine, isoleucine, leucine, tyrosine and phenylalanine. The SCP values for proline, glycine and valine are somewhat higher than the corresponding values for LDL protein. This may be caused by a minor impurity in SCP. Overall, however, the amino acid composition of native SCP is very similar to the amino acid composition of rat serum LDL protein.

4. Discussion

In the present work we have described the purification of native SCP from unheated S_{105}. As we have shown previously [3], SCP incubated alone with [^{3}H]squalene is inert since chemically unchanged substrate is recovered. However, when purified SCP is added to an inactive buffer-washed microsomal preparation from rat liver, we have shown for the first time that it is capable of reconstituting the entire biosynthetic sequence from squalene to cholesterol. We have also achieved successful reconstitution by adding purified SCP to buffer-washed acetone powders of rat liver microsomes.

Furthermore, SCP binds not only water-soluble precursors of cholesterol but also lipid components of lipoprotein, i.e., cholesterol, cholesterol ester, phospholipid and triglyceride. In addition, the amino acid composition of SCP (table 2) is very similar to the amino acid composition of the protein moiety of serum low density lipoprotein [14]. A further examination of the work of Koga et al. [14] shows that the amino acid compositions of serum very low density lipoprotein (VLDL) and high density lipoprotein (HDL) from the rat do not correspond to the amino acid composition of SCP. In a more recent study [15], Scanu and co-workers examined subunit polypeptides from rat serum lipoproteins. Again the amino acid composition of SCP is very similar to their P I low density apolipoprotein which is the predominant (70%) and distinctive component of low density apolipoprotein [15]*. The amino acid compositions of HDL peptides were not consistent with the amino acid composition of native SCP.

These findings are compatible with the novel hypothesis that SCP simultaneously serves both as a carrier for the enzymatic synthesis of cholesterol and as the protein component of LDL, specifying LDL assembly. Thus the enzymatic synthesis by the liver of lipid components (e.g., cholesterol) of LDL may coincide with LDL assembly. It is interesting to note that the half-life in man for the protein moiety of LDL has been reported to be approx. 3.5 days [16]. The half-life for cholesterol in Pool A (which includes serum and liver cholesterol) has been reported to be 3–4 days [17]. The similar half-lives for cholesterol and LDL protein are consistent with our hypothesis. Also, in preliminary experiments [18] we have been able to detect native SCP only in the liver. SCP could not be detected in brain, adrenal, kidney, lung, intestine or spleen. Shah [19] also could not detect SCP in the brain. These observations are also consistent with the above hypothesis since the liver is the principal organ responsible for serum LDL synthesis, while the lung, brain, adrenal, kidney and spleen do not synthesize serum LDL. Also, it seems logical that the enzymatic

* The P I apolipoprotein is also present in VLDL, where it constitutes 25–40% of VLDL protein [15].

conversion of squalene into cholesterol should occur with intermediates and final product (cholesterol) bound to LDL protein since it has been shown that serum LDL carriers 70% of the plasma cholesterol [20].

Certain aspects of the disease abetalipoproteinemia [21–25] may be explained by the hypothesis proposed here. In this condition serum cholesterol concentrations are characteristically very low (e.g., 30–50 mg per 100 ml of serum compared with the normal range of 150–250 mg per 100 ml of serum) [21–24]. It is thought that the protein component of LDL is either absent [21, 22] or not functional [23–25] in these individuals. Our hypothesis would suggest that these individuals may have a marked decrease in cholesterol synthesis by the liver due to absent or defective SCP (LDL protein). This could be a major cause for the observed low serum cholesterol concentration.

Vagelos and co-workers [26] have described the requirement for an *acyl carrier protein* (ACP) in the biosynthesis of fatty acids. There appear to be distinct differences between the roles of ACP and SCP: i) ACP forms a covalent thioester bond while acting as a carrier in fatty acid synthesis. SCP, however, does not form a covalent link, but is held to the substrate by hydrophobic forces. ii) ACP is regenerated after the synthesis of palmitic acid. Our experiments indicate, however, that SCP is not regenerated after the appearance of cholesterol, but instead cholesterol remains bound to SCP and causes it to aggregate and migrate as a lipoprotein on Sephadex G-200 [5]. Thus if SCP serves both as a carrier during cholesterol synthesis, and as the protein component of LDL, specifying LDL assembly, then the biological role of SCP would be very different from the role of ACP in fatty acid synthesis. The whole process of LDL assembly could occur in a multi-enzyme complex in the endoplasmic reticulum of liver, for which we suggest the name lipoprotein synthetase. Viewed in this context SCP would be both a carrier in cholesterol synthesis and a substrate in LDL assembly by the liver.

In recent preliminary communications [27–29] Ritter and Dempsey have reported the purification of a heat-stable peptide isolated after heating S_{105} to 100° for 5 min. They believe that this peptide is SCP [28, 29], based on stimulation of the conversion of 7-dehydrocholesterol into cholesterol. They also report that this reaction is stimulated by a peptide, R-Gln-II, which was isolated from lipid-extracted high density lipoprotein. It was necessary in both cases to preincubate the sterol substrate with the peptide, in order to obtain stimulation of the conversion of 7-dehydrocholesterol into cholesterol. These results are in marked contrast to our consistent demonstration that native SCP is heat-labile, either in crude extracts or in the purified state [3, 7, 30]. Other investigators have also noted that the ability of S_{105} to stimulate various reactions in cholesterol biosynthesis is destroyed by heating [31, 32]. Also, we have been unable to detect the peptide isolated by Ritter and Dempsey in unheated S_{105} [7]. Thus the origin of this peptide is in doubt. Recent preliminary observations in our laboratory [30] suggest that the peptide isolated by Ritter and Dempsey after heating S_{105} does not originate from native SCP at all, but instead originates from breakdown of a protein fraction which has no SCP activity prior to heating. Another difference is that native SCP does not require preincubation with substrate (in fact, preincubation is inhibitory) either in crude or purified form.

In any event the amino acid composition, electrophoretic mobility, heat lability, and lack of a requirement for preincubation all indicate that native SCP isolated from unheated S_{105}, as reported here, is clearly a different protein than the material isolated by Ritter and Dempsey. We conclude that the protein isolated as described in the present report represents the protein which operates physiologically.

If SCP is the protein component of LDL, then our isolation of this protein is, to the best of our knowledge, the first isolation and characterization of a protein component of lipoprotein *before* the attachment of lipid. Because of the absence of lipid, very gentle techniques of protein purification can be employed, thus facilitating studies concerned with the biological activity and quaternary structure of this protein. This is a considerable advantage since methods used for the isolation of the protein moieties of serum lipoproteins presently depend on rigorous solvent extraction along with the use of substances such as 8 M urea to prevent aggregation [15].

Acknowledgements

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