Purification and Properties of a Soluble Protein Activator of Rat Liver Squalene Epoxidase*

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A soluble rat liver protein, termed "supernatant protein factor" (SPF), that stimulates microsomal squalene epoxidase has been purified approximately 11,000-fold. The most highly purified preparation obtained by isoelectric focusing shows a single coincident peak for activity and protein (the isoelectric point, pi, was 6.74). SPF is about 95% pure, judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and it migrates to a position corresponding to an apparent molecular weight of 47,000. An amino acid analysis of SPF is presented, and the properties of SPF and of the various soluble protein activators of microsomal sterol biosynthesis described by other laboratories are compared.

It has been known for some time that the NADPH- and O2-dependent conversion of squalene to lanosterol by liver microsomes is enhanced by the addition of 105,000 x g supernatant (1). After squalene-2,3-oxide was demonstrated to be an intermediate in the squalene-lanosterol conversion (2, 3), supernatant dependence was found to be associated with squalene epoxidase, a microsomal activity distinct from squalene-2,3-oxide cyclase (4). One of the soluble components that stimulated squalene epoxidase was a heat-labile supernatant protein, termed "supernatant protein factor," it was purified 84-fold and had an apparent molecular weight of 44,000 (5). We here report the purification of SPF to apparent homogeneity and show that it is a single polypeptide chain with a molecular weight of 47,000.

EXPERIMENTAL PROCEDURES

Materials

Female rats, strain CD, were obtained from Charles River Breeding Laboratories; 101- to 125-g rats were used for the biosynthesis of [14C]squalene, while 201- to 225-g rats were the source of liver microsomes and SPF. Na-[2-14C]Mevalonic acid dibenzylohexylenediamine salt, 7.98 Ci/mmol, was from New England Nuclear. The following were products of Sigma (catalog numbers are listed when more than one grade is sold): ATP (No. A3127), glucose-6-phosphate (No. G7879), glucose-6-phosphate dehydrogenase (No. G8878 from Torula yeast), Tween 80, Tris (No. T1505), Triton-HCI, FAD, diethiothreitol, 2-mercaptoethanol (No. M6250), bromophenol blue, catalase (beef liver No. C-100), L-glutamic dehydrogenase (bovine liver No. G2626), aldolase (rabbit muscle No. A6253), and NAD+ (No. N7004). NADPH and phosphatidylserine (bovine No. 6589) were from P-L Biochemicals. Calbiochem supplied Amo-1618, lumarase (heart muscle), and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle). Amo-1618 was recrystallized from absolute methanol/anhydrous ether by trituration with ether (m.p. 157-158°, corrected). Phosphatidylglycerol (egg lecithin) was purchased from Serdy Research Laboratories. Amides black 10B was from Canaco, amylase (Ampholine, pH 6 to 8) was from LKB, carbonic anhydrase was from Worthington, and constant-boiling HCI (Sequential grade) was from Pierce. Bovine serum albumin (No. 90 2506) and ovalbumin were obtained from Schwarz/Mann. Bio-Rad supplied SDS, Coomasie brilliant blue R-250, DEAE-cellulose (0.7 meq/g), and phosphocellulose Sephadex G-25 (medium), G-75, and G-50 (fine) were obtained from Pharmacia, and the Quantum LQED thin layer plates were from Kontes. Chemicals not specifically listed above were standard reagent grade.

Methods

Biosynthesis of [14C]Squalene—Except in three details, [14C]squalene was prepared enzymatically from Na-[2-14C]mevalonic acid by the method of Popjak (6). Livers were homogenized directly in a Thomas 3431-K18 Potter-Elvehjem tissue grinder, size B8; cofactors (NADPH substituted for NADP+) were titrated to pH 7 with NaOH; and the reaction mixture was made 0.3 unit/ml in glucose-6-phosphate dehydrogenase to supplement the endogenous NADPH-regenerating system. A typical reaction containing 50 μCi (1.7 mg) of Na-[2-14C]mevalonic acid dibenzylohexylenediamine salt yielded 6.2 μCi (0.18 mg) of purified [14C]squalene; the product displayed a single peak of radioactivity on gas chromatography (F & M model 400 gas chromatograph, Packard model 884 proportional counter, 12' UV-17 columns, oven temperature 290°) that co-chromatographed with authentic squalene. [14C]Squalene was diluted with unlabeled squalene to a radiospecific activity of 0.25 Ci/mmol and was stored frozen as a 4 mM solution in benzene.

Activity Assays—SPF activity was assayed under conditions similar to those described previously (5). Amo-1618, a plant growth inhibitor (7), was added to the assay mixtures to inhibit squalene-2,3-oxide cyclase (8). [14C]Squalene (40 nmol, 0.25 Ci/mmol in benzene) and 50 μg of Tween 80 in 50 μl of acetone were mixed, and the solvents were evaporated under nitrogen. Along with substrate and detergent, the reactions contained, in a volume of 1 ml, 0.1 M Tri/HCl buffer, pH 7.3 at 37°; 1 mM EDTA; 0.3 mM Amo-1618; 0.01 mM FAD; 0.1 mg of phosphatidylglycerol per ml; 1 mM NADPH; 1.28 mg of microsomal protein per ml; and various concentrations of SPF fractions, as noted. Mixtures were incubated at 37° for 30 min, and products were isolated and assayed as described (5), except that Quantum LQED (19-channel) precoated silica gel plates (20 × 20 cm) were used for product isolation.

Unit of SPF Activity—Squalene epoxidase activity was calculated after correcting for the recovery of total radioactivity (usually about 70%). One unit of SPF activity is defined as 1 incremental nmol of squalene-2,3-oxide produced in 30 min under standard assay condi-
Soluble Protein Activator of Squaleone Epoxidase

The concentration of Tris/HCl in the G-75 fraction was reduced to 0.05 M for convenient ultrafiltration; dialyzed in the same apparatus with 39 ml of a 50 mM Tris/HCl, 50 mM KCl, 0.5 mM dithiothreitol buffer (pH 7.5); and incubated in the presence of Strikantaiah et al. (9); incubations were performed as described except that 1-ml assay volumes and 40 nmol of squaleone were used. Products were isolated and assayed as described in epoxidas assays above. One unit of SCP, activity is defined as 1 increment of the fast sediment at 280 nm of 0.660 liters g⁻¹ cm⁻¹ for the standard protein (12).

Preparation of Microsomes and Supernatant Fractions - Microsomes and supernatant fractions from 207 female rat livers were prepared as previously described (4), with the following modifications. Rats were anesthetized with diethyl ether before decapitation, and after perfusion the livers were homogenized at 0°C in a Thomas 3431-E55 grinding vessel (size C, Potter-Elvehjem type) with three passes of 3431-E55, 3431-E55, and Teflon pestle rotating at 1000 rpm. Low speed centrifugations were performed at 13,000 × g, and high speed centrifugations were performed at 78,100 × g. The high speed supernatant (S₁₀₀) was frozen at -70°C and the microsomes were washed twice with 0.1 M Tris/HCl buffer, pH 7.5, resuspended in a volume equivalent to the weight of the liver and frozen at -70°C in 0.5-ml aliquots. The final protein concentration of the microsome suspension was 25.6 mg/ml.

Purification of SCP - The first three steps were based on those reported earlier (5). Acetone fractions were performed with 300 ml of -70°C acetone and 30°C acetone, the same buffer was used with the aid of a Teflon pestle and then centrifuged at 4°C for 10 min (12,000 × g) to remove particulate matter. Amino acid analyses were performed as previously described (5). Acetone was removed by immediately applying the supernatant to a column of Sephadex G-25 (medium) (5.8 x 66 cm) and a bed volume of that buffer equivalent to one-third of the original liver volume. The column was drained at 2 ml/min, and the coincidence of SCP activity and the single protein peak was dominated at pH 7.5.

Amino Acid Analysis - Protein samples (focused fraction, 75 μg) were dialyzed against deionized water before transfer to constant-boiling HCl. Analyses were performed on a Durrum D500 instrument fitted with a column of Dowex DC-4A (0.175 x 45 cm), 8-μm bead cation exchanger in the laboratory of Dr. Lisa Steiner at the Massachusetts Institute of Technology.

RESULTS

While SCP activity co-chromatographed with a protein peak on the Sephadex G-75 column, subsequent chromatography on DEAE-cellulose (Fig. 1) clearly indicated that SCP accounted for only a small fraction of the protein in the G-75 fraction. SCP was held only tenuously to the DEAE column under the conditions described and began to elute with the wash buffer. The beginning of the KCl gradient removed the rest of the SCP activity from the column. Preparative isoelectric focusing of the DEAE fraction in pH 6 to 8 gradients resulted in more effective separation of SCP from inactive protein than pH 5.3 to 10 systems; a single major peak of absorbance at 280 nm was observed by the coincidence of SCP activity and the single protein peak.
Soluble Protein Activator of Squalene Epoxidase

**Fig. 1.** DEAE-cellulose chromatography of the G-75 fraction as described under "Experimental Procedures." ○, absorbance at 280 nm; □, pH; ▲, SPF activity of 20-μl aliquots. Activity is expressed as incremental counts per min of squalene-2,3-oxide recovered from assay mixtures incubated under standard conditions in the presence of SPF over controls without SPF. Not shown is the large amount of inactive protein that routinely eluted behind the peak of SPF activity.

**Fig. 2.** Isoelectric focusing of the DEAE fraction on a pH 6 to 8 gradient as described under "Experimental Procedures." ○, absorbance at 280 nm; □, pH; ▲, SPF activity of 5-μl aliquots. In a corresponding focusing experiment without protein, the batch of ampholytes used produced background absorbances at 280 nm of 0.085 to 0.13 in the pH 6 to 8 region.

In the effluent of the high resolution isoelectric focusing step (Fig. 2) indicated homogeneity with respect to isoelectric point. SDS-polyacrylamide gel electrophoresis of 4.6 μg of the focused fraction (Fig. 3A) showed a major polypeptide band at RF, 0.46, with minor bands at RF, 0.57 and 0.70. According to a scan of the gel at 550 nm (Fig. 3B), the major band comprised 95% of the total. A similar electrophoretic analysis of 9.1 μg (not shown) indicated that the minor bands constituted 2.9 and 1.7% of the total absorbance, respectively.

When SPF was run on SDS-polyacrylamide gels along with known protein standards (Fig. 4), migration of the protein corresponded to a molecular weight of approximately 47,000 for the polypeptide chain. Amino acid analyses of pure SPF are shown in Table II.

**Fig. 3.** SDS-polyacrylamide gel electrophoresis of 4.6 μg of SPF (focused fraction). The discontinuous buffer ("SDS-disc") system of Maizel (14) was employed; details are given under "Experimental Procedures." A, photograph of Coomassie-stained gel; B, scan of same gel at 550 nm.

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**Table I**

| Fraction | Volume | Total Protein (mg) | Total Activity (units) | Yield of Activity (%) | Specific Activity (units/mg) | Purification Factor |
|----------|--------|--------------------|------------------------|----------------------|-----------------------------|-------------------|
| S,        | 2460   | 73,800             | 10,600                 | 100                  | 0.143                       | 1                 |
| Acetone   | 866    | 11,400             | 13,700                 | 129                  | 1.20                        | 8.39              |
| Combined  | 50     | 2,850              | 23,750                 | 224                  | 8.56                        | 62.6              |
| DEAE      | 7.0    | 18.0               | 12,800                 | 121                  | 7.10                        | 4960              |
| Focused*  | 4.0    | 1.82               | 2,820                  | 26.6                 | 1500                        | 10,800            |

* Corrected; only one-half of the material was processed through this step.

**Fig. 4**. When SPF was run on SDS-polyacrylamide gels along with known protein standards (Fig. 4), migration of the protein corresponded to a molecular weight of approximately 47,000 for the polypeptide chain. Amino acid analyses of pure SPF are shown in Table II.

**TABLE II**

| Amino Acid | SPF | Standard |
|------------|-----|----------|
| Alanine    |     |          |
| Aspartic  |     |          |
| Aspartate |     |          |
| Asparagine|     |          |
| Cysteine  |     |          |
| Glutamic  |     |          |
| Glutamate |     |          |
| Glycine   |     |          |
| Histidine |     |          |
| Isoleucine|     |          |
| Isoleucine|     |          |
| Lysine    |     |          |
| Methionine|     |          |
| Phenylalanine|  |          |
| Proline   |     |          |
| Serine    |     |          |
| Threonine |     |          |
| Tryptophan|     |          |
| Tyrosine  |     |          |
| Valine    |     |          |

**DISCUSSION**

The procedure described here affords a homogeneous SPF preparation after approximately 11,000-fold purification. Judged from SDS-polyacrylamide electrophoresis, the squalene epoxidase-stimulating protein consists of a single 47,000-dalton polypeptide chain. In a previous report the native molecu-
Differentiate SCP, from SPF, under the conditions for SCP, assay sizes and the dimeric subunit structure would seem to differ-
activity is defined as an entity that stimulates a two-step
equilibrium) and 13,000 for the subunit (sedimentation
weight used to calibrate the gel system included bovine serum albumin
molecular weight was compared to 11,000 for SPF, and carbonic anhydrase
positions of the standard proteins presented are the
results of two identical experiments, and the RF of SPF indicated is
the average of four determinations.

**TABLE II**
Amino acid compositions of supernatant protein factor and squalene
and sterol carrier protein

| Amino acid     | Best normalized value/μg of SPF | Moles % SPF | Moles % squalene and sterol carrier protein | Residue or 47,000 g SPF |
|----------------|--------------------------------|-------------|---------------------------------------------|-------------------------|
| Asx            | 8.22                           | 9.49        | 9.63                                        | 40                      |
| Threonine      | 3.54                           | 4.09        | 6.94                                        | 17                      |
| Serine         | 5.50                           | 6.35        | 4.17                                        | 27                      |
| Gia            | 11.4                           | 13.2        | 11.3                                        | 55                      |
| Proline        | 4.88                           | 5.64        | 3.42                                        | 24                      |
| Glycine        | 7.02                           | 8.11        | 9.91                                        | 34                      |
| Alanine        | 4.72                           | 5.45        | 3.24                                        | 23                      |
| 1/2 cystine    | 1.86                           | 2.15        | 3.06                                        | 9                       |
| Valine         | 4.65                           | 5.37        | 7.87                                        | 22                      |
| Methionine     | 2.06                           | 2.38        | 4.35                                        | 10                      |
| Isoleucine     | 3.32                           | 3.83        | 6.48                                        | 16                      |
| Leucine        | 8.81                           | 10.2        | 6.02                                        | 43                      |
| Tyrosine       | 5.55                           | 4.16        | 3.93                                        | 17                      |
| Phenylalanine  | 3.48                           | 4.02        | 5.46                                        | 17                      |
| Histidine      | 2.90                           | 3.35        | 1.48                                        | 14                      |
| Lysine         | 6.91                           | 7.98        | 11.1                                        | 33                      |
| Arginine       | 3.72                           | 4.30        | 2.04                                        | 18                      |

* Data for rat squalene and sterol carrier protein adapted from Ref. 19.
* Average of three hydrolysates.
* Average of three hydrolysates (no destruction noted between 20-
and 70-hydrolysates).
* Average of three hydrolysates (no interference from cysteine noted).
* From hydrolysis of performic acid-oxidized sample.
* Seventy-hour value.
* Calculated from 20- and 70-h data according to Moore and Stein (15).
* Average of 20- and 70-h data.

process, squalene epoxidation and squalene epoxide-lanosterol cyclization. Therefore, SCP, may affect either or both of the
two steps. We have demonstrated that SPF stimulates squalen
epoxide-dimerization, the first of the two events. If we assume that
the two steps are activated by separate proteins, our results
would be consistent with the view that SCP, affects the subsequent
step, *i.e.* the squalene epoxide-lanosterol cyclization.

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J.B. Ferguson and K. Bloch

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