Rat hepatic microsomal squalene synthetase (EC 2.5.1.21) was induced 25-fold by feeding rats with diet containing the hydroxymethylglutaryl-coenzyme A reductase inhibitor, fluvastatin, and cholestyramine, a bile acid sequestrant. A soluble squalene synthetase protein with an estimated mass of 32–35 kDa, as determined by gel filtration chromatography on Sephacryl S-200 column, was solubilized out of the microsomes by controlled proteolysis with trypsin. Approximately 25% of the activity was recovered in a soluble form. The enzyme was purified to homogeneity utilizing a series of column chromatography purification steps on DEAE-cellulose, hydroxylapatite, and phenyl-Sepharose sequentially. The purified enzyme showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Initial kinetic analysis indicated an \( V_{\text{max}} \) values for trans-farnesyl diphasphate of 1.0 \( \mu \text{M} \) and for NADPH of 40 \( \mu \text{M} \). The \( V_{\text{max}} \) with respect to trans-farnesyl diphasphate was calculated at 1.2 \( \mu \text{mol/min/mg} \), NADPH also serves as substrate for the reaction with \( S_{0.5} \) value of 800 \( \mu \text{M} \). Western blot analysis utilizing rabbit antisera raised against the purified, trypsin-truncated enzyme showed a single band for the isolated solubilized enzyme at 32–33 kDa and a band for the intact microsomal enzyme at about 45–47 kDa.

The formation of squalene from trans-farnesyl diphasphate (FPP) is catalyzed by squalene synthetase (farnesylphosphatase:farnesylphosphatase farnesyltransferase, EC 2.5.1.21) in two separate steps. The first is the condensation of two molecules of FPP to form an intermediate presqualene diphasphate through a unique dimerization reaction (Rilling and Epstein, 1969; Poulter and Rilling, 1981). This intermediate is then reductively rearranged in a second step for the reaction to form squalene using a reduced pyridine nucleotide as a reductant.

Squalene synthetase is localized at a branch point in the mevalonate pathway. The substrate for the reaction, FPP, is not only utilized for the production of sterols and their products but also serves as substrate for other prenyl transferases. Thus, repetitive cis additions of isopentenyl diphasphates result in the formation of 2,3-dehydrodolichyl diphasphate, whereas trans polymerization results in nonaprenyl diphasphate, an intermediate in ubiquinone synthesis.

The identification of Fludorotamine A-farnesylated mating factor (Kamiya et al., 1979) and the observation that mevalonate-derived products are incorporated into cellular proteins (Schmidt et al., 1984) led to the identification of a number of thioether-linked farnesyl and geranylgeranyl moieties with proteins (Goldstein and Brown, 1990; Glomset et al., 1990). Some of these prenylated proteins such as the ras proteins play a major role in regulation of normal cellular processes and in oncogenesis. The localization of squalene synthetase in this central branch point of the mevalonate pathway, and the observation that its activity is suppressed in cultured fibroblasts grown in the presence of low density lipoproteins led to the hypothesis that its regulation plays a major role in the diversion of the flux of intermediates to either the sterol or the nonsterol pathway (Faust et al., 1979).

The reaction catalyzed by squalene synthetase connects the two cellular compartments within which the sterol biosynthetic pathway is localized. FPP is produced from mevalonate through a sequence of reactions which utilize and produce cytosolic intermediates whereas squalene and all subsequent intermediates in the production of sterols are localized in the endoplasmic cell membrane. The hepatic enzyme has not yet been purified, and the number of proteins required for the two-step reaction is not known. Yeast has been the organism of choice for the studies and purification of squalene synthetase. Early attempts to solubilize this enzyme employed deoxycholate as detergent (Shechter and Bloch, 1971). This solubilization was later repeated by Agnew and Popjak (1978) using the same detergent. The yeast-derived enzyme was finally purified to homogeneity by the use of a mixture of nonionic detergents and chromatography purification (Kuwik-Rabiega and Rilling, 1987; Sasiak and Rilling, 1988). The purified yeast enzyme is a single polypeptide of \( M_0 \), 47,000, and it catalyzes the synthesis of squalene from FPP via presqualene diphasphate. Recently, the molecular cloning of the yeast squalene synthetase gene was achieved either by functional complementation of a squalene synthetase-deficient mutant (Jennings et al., 1991) or by screening a genomic library (Handler et al., 1991).

We report here a technique for the proteolytic release of a truncated, soluble, active fragment of rat hepatic microsomal squalene synthetase and its purification to homogeneity without the use of detergents. The catalytic and physical properties of this soluble enzyme are discussed as well.
EXPERIMENTAL PROCEDURES

Materials

Radiolabeled Compounds—In early experiments FPP was synthesized in one of our laboratories (15). Trans-[3H]farnesol (32.6 mCi/mmol) was prepared according to the method of Secher and Bloch (17) and was pyrophosphorylated by the method of Poppak (1969). Trans-[3H]farnesyl dipiphosphate was obtained according to the method of Gafni and Secher (1979). The experimental results obtained by the use of this radiolabeled substrate are reported in dpn of radioactivity. In later experiments, [3H]FPP (300,000 disintegrations/minute of 1-[3H]farnesyl dipiphosphate, triammonium salt, 143 mCi/mmol) was purchased from New England Nuclear. [2-14C]Mevalonate (51 mCi/mmol) and radioiodinated 2-14C-HMG-CoA (R) was purchased from the Radiochemical Centre, Amersham Corp. [14C]Squalene was biosynthesized in our laboratory from [14C]mevalonate according to the method of Poppak (1969) with slight modifications (Secher and Paleg, 1972). Specific radioactivity for both labeled squalene and 2,3-oxidosqualene was determined by thin-layer chromatography.

Non-radiolabeled Materials—The following materials were purchased: Sigma (NAD, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 5-bromo-4-chloro-indolyl phosphate, nitroblue tetrazolium, 4-chloro-1-naphthol, HMG-CoA, mevalonic acid, bovine serum albumin, SDS, Comassie Brilliant Blue R and G, cholestyramine, Amido Blue-Black, and Ponceau S. Phenyl-Sepharose 4B-CL was obtained from Pharmacia LKB Biotechnology Inc. and DE52 from Whatman. Goat anti-rabbit alkaline phosphatase conjugate, avidine alkaline phosphatase conjugate, and precasted molecular mass standards were obtained from Bio-Rad. Lovastatin was a gift of Dr. A. Alberts from Merck, Sharp and Dohme Laboratories. Flavusatin (Sandoz compound XU 62-320) was provided by Sandoz Pharmaceutical. Nitocellulose paper was purchased from Gelman or Bio-Rad. Non-radioactive FPP was provided by Dr. Larry Perez, Sandoz. Other chemicals and reagents were of analytical grade and purchased from commercial sources.

Methods

Preparation of Normal and Squalene Synthetase-induced Rat Liver Microsomes—Male 170–190 g Wistar Charles River rats were fed a powdered normal Purina rat chow diet and kept on an altered light cycle from 7 p.m. to 7 a.m. (normal rats). After 7–10 days, the rats were fed the diet with 2% (w/v) of 2,3-oxidosqualene for 3 days followed by the powdered diet plus 5% cholestyramine and 0.15% of either lovastatin (LO diet) or flavusatin (FL diet) for 3 more days. The rats were then sacrificed; their livers were removed and quickly cooled in saline. The remaining purification procedures were carried out at 4 C. The livers were blotted, weighed, minced, and washed in 2 volumes (2 ml of buffer/g of tissue) of buffer A: containing 0.3 M sucrose, 10 mM HEPES, pH 7.4, 1.0 mM DTT, 1.0 mM EDTA (buffer A). When necessary, the protease inhibitors 1.0 mM PMSF, 10 mM leupeptin, and 1.0 mM pepstatin (hereafter called protease inhibitors) were added to buffer A, as mentioned under "Results." 2 volumes of buffer A were added, and the livers were homogenized with a Polytron and then with a moderately tight-fitting Teflon/glass Elvehjem homogenizer. After centrifugation of the homogenate for 5 min at 3,000 rpm in a Sorval SS-34 rotor, the supernatant (96 ml) was diluted by 500 ml of buffer containing 0.3 M sucrose, 10 mM HEPES, pH 7.4, 1.0 mM DTT, 1.0 mM EDTA (buffer A). When necessary, the protease inhibitors 1.0 mM PMSF, 10 mM leupeptin, and 1.0 mM pepstatin (hereafter called protease inhibitors) were added to buffer A, as mentioned under "Results." 2 volumes of buffer A were added, and the livers were homogenized with a Polytron and then with a moderately tight-fitting Teflon/glass Elvehjem homogenizer. After centrifugation of the homogenate for 5 min at 3,000 rpm in a Sorval SS-34 rotor, the speed was increased to 9,000 rpm and continued for 15 min. The surface lipids were then aspirated, and the supernatant removed and recentrifuged at 105,000 X g for 30 min. After aspiration of surface lipids, the microsomes were again resuspended in buffer B to a volume equal to the original total liver weight and frozen in portions at -80 C. The S90 preparation was centrifuged in an Airfuge (Beckman) for 10 min at 160,000 X g (S90) before it was used for assay. The solubilization of liver microsomal preparations was stored in liquid N2.

Freeze-Thaw Solubilization of Microsomal Squalene Synthetase—Microsomes prepared without protease inhibitors were homogenized with a glass/Teflon homogenizer. The suspension was frozen in liquid N2 for 10 min, thawed at 37 C, and further incubated at 37 C for 10 min. During that incubation the microsomal suspension was homogenized twice. The resulting suspension was centrifuged at 105,000 X g for 1 h. The freeze-thaw supernatant extract was separated from the microsomes and stored at -80 C. The microsomal pellet was resuspended in the same buffer and freeze-thaw treated as described above for an additional four to five cycles.

Tryptsin Solubilization of Squalene Synthetase from Microsomes—125 ml of frozen FL microsomes, prepared in the presence of protease inhibitors, (protease concentration = 17.6 mg/ml) were thawed, diluted 1:1 with 20 mM potassium phosphate, 2.0 mM DTT, adjusted to pH 7.6, and centrifuged for 30 min at 105,000 X g. The pellet was suspended in 10 mM potassium phosphate, pH 7.6, with 1.0 mM DTT, homogenized with a glass/Teflon Elvehjem homogenizer, and the solubilized material was adjusted to 125 ml (protein concentration = 12.2 mg/ml). Tryptsin, Sigma Type XIII, 1:1 tosylamide-2-phenylethylchloromethyl ketone-treated (7.6 mg in 1.0 ml of 1.0 mM HCl) was added, and the sample was incubated at 15 C for 2 h with occasional stirring. To monitor the progress of the release of squalene synthetase during the trypsinization procedure, 1 ml samples were removed at 0, 1, and 2 h, and 20 ml of soybean trypsin inhibitor (SBTI, 38.1 mg in 2.50 ml of 1.0 mM HCl) was added to give a 5-fold excess of SBTI compared to trypsin. Portions of the samples of the various time periods were centrifuged for 5 min at 160,000 X g in a Beckman airfuge prior to assay. After 2 h of trypsination, 2.44 ml of SBTI solution was added to the remaining 122 ml of the suspended microsomes followed by centrifugation for 30 min at 105,000 X g. Protease inhibitor mixture was added to the S90 supernatant (96 ml) to a final concentration of 1.0 mM PMSF, 10 mM leupeptin, and 1.0 mM pepstatin, and the pH was adjusted 8.0. This trypsin-solubilized enzyme preparation was used for further purification.

DEAE-Cellulose Ion-Exchange Chromatography—150 g of Whatman DE52 anion-exchange cellulose was equilibrated with 100 mM potassium phosphate, pH 8.0, and packed into a 2.6 X 55-cm Pharmacia C10 column. The column was washed with 10 mM potassium phosphate, pH 8.0, containing 2.5% ethylene glycol. The column was then eluted with 500 ml of buffer containing 10 mM potassium phosphate, pH 8.0, 1.0 mM DTT, 1.0 mM EDTA and 25% ethylene glycol (buffer C) followed by 100 ml of buffer C plus protease inhibitors. This and subsequent chromatography was performed using a Pharmacia FPLC system controlled by an LCC500 plus liquid chromatography controller. The 96 ml S90 of trypsin-solubilized enzyme was applied onto the DE52 column using a peristaltic pump at a flow rate of approximately 1 ml/min. From this point on, all buffers used for the purification of the enzyme contained protease inhibitors. The sample was eluted from the column at 1.0 ml/min with first washing with 500 ml of buffer containing 5% ethylene glycol followed by 0.8 M NaCl to 0.5 M potassium phosphate, 280 nm, and squalene synthetase was eluted at 1.0 ml/min. Fractions containing the peak of activity (~0.15-0.25 mCi NaCl) were pooled for separation on a hydroxyapatite column.

Hydroxyapatite Chromatography—A 1.6 X 28 cm Pharmacia C10 column was packed with hydroxyapatite (Bio-Rad Bio-Gel HTP) and equilibrated in buffer D (10 mM potassium phosphate, pH 6.8, 1.0 mM DTT and 20% ethylene glycol). The 75 ml pool of active fractions from the DE52 column was applied to the hydroxyapatite column using a peristaltic pump at a flow rate of approximately 1 ml/min. The enzyme was eluted from the column at 1.0 ml/min with 150 ml of Buffer D followed by a linear salt gradient of 600 ml up to 0.5 M potassium phosphate. Fractions of 10 ml were collected. Elution was monitored for protein at 280 nm, and squalene synthetase was assayed for activity. Fractions containing the peak of activity (~0.22-0.24 mM potassium phosphate) were pooled for separation on phenyl-Sepharose.

Phenyl-Sepharose Chromatography—A 1 X 8 cm Pharmacia C10 column was packed with phenyl-Sepharose (Pharmacia) and equilibrated in buffer E (20 mM potassium phosphate, pH 7.4, 1.0 mM DTT, 1.0 mM EDTA, and 20% ethylene glycol). The 18.5 ml pool of the most active hydroxyapatite fractions was diluted 1:1 with 40% saturated ammonium sulfate solution at pH 7.4 to give 20% saturation in ammonium sulfate and approximately 12.5% ethylene glycol. The diluted sample was applied to the phenyl-Sepharose column (1.6 X 20 cm) with linear salt gradient of 200 ml of Buffer E (high salt) followed by a 40 ml decreasing salt and increasing ethylene glycol linear gradients to a final buffer solution containing 20 mM potassium phosphate, pH 7.4, 1.0 mM DTT, 1.0
mm EDTA, and 50% ethylene glycol (buffer G). Elution continued with an additional 20 ml of buffer G, and fractions of 1.0 ml were collected. Elution was monitored for protein at 280 nm, and enzymatic activity was determined for the various fractions. Fractions containing the peak of activity were pooled and concentrated to one-fifth the volume using an Amicon ultrafiltration apparatus with a PM-10 membrane.

Superose 12 Chromatography—When necessary, purified squalene synthetase obtained from the phenyl-Sepharose column was further purified on Superose 12. A 30-cm Superose 12 column (Pharmacia) was equilibrated with buffer H (20 mM potassium phosphate, pH 7.4, 1.0 mM DTT, 1.0 mM EDTA, 25% ethylene glycol, and 500 mM MgCl2). 120 µl of the ultrafiltration concentrate of the active fractions eluted from the phenyl-Sepharose was diluted to 300 µl with buffer H, 200 µl of the diluted enzyme were applied to and chromatographed through the Superose 12 column at 0.25 ml/min. Fractions of 0.2 ml were collected. Elution was monitored for protein at 280 nm, and enzymatic activity was determined with the microwell assay described below.

Enzymatic Assays—Assays for HMG-CoA reductase, squalene epoxidease, and 2,3-oxidosqualene-lanosterol cyclase were performed as described earlier (Eilenberg et al., 1989). Squalene synthetase was assayed as follows. Reactions were carried out in 96-well V-shaped polystyrene microtitration plates. The reactions in total volume of 100 µl contained 100 mM potassium phosphate, pH 7.4, 5.0 mM MgCl2, 5.0 mM CHAPS, 10 mM DTT, 2.0 mM NADPH, and 10 µM [3H]FPP. Enzyme concentrations in the assays were adjusted so that less than 10% conversion of the substrate was catalyzed. Incubations were performed in covered plates for 20 min at 37 °C. The reaction was stopped with 10 µl of 1.0 M EDTA, pH 9.2. In addition, 10 µl of unlabeled 0.5% squalene in ethanol was then added as carrier. 60 µl of the reaction (50% of the final volume) were applied onto analytical 1.8 × 20-cm, 250-µm thick, Silica Gel-G thin layer chromatography plates which were developed by 20-cm, 250-µm thick, Silica Gel-G thin layer chromatography plates which were developed by 20-cm, 250-µm thick, Silica Gel-G thin layer chromatography plates which were developed by 7.3 volatile solvents. Radioactivity in zones containing the squalene (Rf = 0.5) were scraped and counted after elution in toluene. The enzymatic activity was reported either in disintegration/minute or in nanomoles of squalene synthetase activity for the purpose of the purification of the enzyme. Animals fed with diet containing 3% cholesterol showed 57% of liver squalene synthetase activity compared to that of rats fed normal diet.

Development of Multiwell Plate Microassay for Squalene Synthetase

Effect of Detergent—We have studied the effect of a variety of detergents on the activity of microsomal squalene synthetase. It was observed that all detergents tried (non-ionic, ionic, and zwitterionic, data not shown), CHAPS was the most effective. Microsomal squalene synthetase activity increased about 6-fold in the presence of 5 mM CHAPS (Fig. 2).

Activation of Sepharose 4B Column Filtered Microsomes by DTT—DTT has little effect on squalene synthetase activity in freshly isolated rat liver microsomes. However, Sepharose 4B column-filtered microsomes which showed little or no enzyme activity can be activated in the presence of 10 mM DTT. The highest activity is observed for enzymes which are prepared and assayed in this concentration of thiol (Fig. 3). This effect is similar to the activation of latent HMG-CoA reductase by DTT (Dotan and Shechter, 1982).

On the basis of these two observations, a microassay of squalene synthetase in a total volume of 100 µl was developed as described under “Experimental Procedures.” One advantage of this microassay is its efficiency in the simultaneous assay of many samples. Samples can be analyzed in a relatively short time in comparison to the traditional assays in

RESULTS

Induction of Rat Hepatic Squalene Synthetase Activity by HMG-CoA Reductase Inhibitors

Feeding rats a diet containing HMG-CoA reductase inhibitors in combination with the bile sequestrant, cholestyramine, results in marked elevation of squalene synthetase activity. At enzyme protein concentrations of up to 60 µg/ml, it was observed that animals fed LO diet had an 11-fold elevation of hepatic squalene synthetase activity, whereas animals maintained on a FL diet had more than 25-fold elevation of hepatic squalene synthetase activity in comparison to the hepatic activity of squalene synthetase in animals maintained on normal diet (Fig. 1). Therefore, we chose the FL diet treatment for induction of rat hepatic squalene synthetase activity for the purpose of the purification of the enzyme. Animals fed with diet containing 3% cholesterol showed 57% of liver squalene synthetase activity compared to that of rats fed normal diet.

Fig. 1. Effect of different diets on the activity of rat liver squalene synthetase. Microsomes were prepared from livers of male Wistar Charles River rats maintained on normal Purina Chow (●), chow supplemented with 3% cholesterol (○), LO diet (△), and FL diet (▼) as described under “Experimental Procedures.” Microsomes were prepared in the presence of a mixture of protease inhibitors and assayed for squalene synthetase activity for 30 min at various protein concentrations. Radiolabeled [3H]FPP of 32.6 mCi/mmol was used for the enzyme assay, and the results are expressed in dpm squalene produced.
which squalene is extracted by organic solvents or radiolabeled protons released during the reaction are exchanged into methanol and distilled (Poppjak, 1969; Kuswik-Rabiega and Rilling, 1987). This procedure was essential for monitoring the activity observed was not residual contamination of microsomes in the soluble preparation. Of the four tested enzymes, only HMG-CoA reductase is known to exist in a soluble truncated form with subunit size of 52-56 kDa as well as in a microsomal form with a subunit size of 97 kDa (Ness et al., 1981). Unlike the activity of squalene epoxidase and 2,3-oxidosqualene-lanosterol cyclase, which are strictly in the microsomes, the activity of squalene synthetase is observed in the S100 fraction as well as in the microsomes, as was also observed for HMG-CoA reductase. This difference in distribution of activities between the cyclase and epoxidase on one hand and squalene synthetase and HMG-CoA reductase on the other strongly pathway, we have observed persistently the existence of squalene synthetase activity in the S100 cytosolic fraction of the preparations. Formation of radiolabeled squalene from either [14C]mevalonate or [3H]farnesyli diphosphate in the soluble preparation persisted even when the separation of the soluble cytosolic fraction from the microsomes was performed at a higher speed of centrifugation (160,000 x g) suggesting that the activity observed was not residual contamination of microsomes in the soluble preparation. In addition, unlike the reaction in microsomes, formation of squalene from [3H]FPP and NADPH in the solubilized S100 enzyme was not accompanied by formation of radiolabeled 2,3-oxidosqualene or steroids (Fig. 4B). The above observations prompted us to examine the existence of squalene synthetase in the soluble extract more carefully in order to determine if the activity observed is catalyzed by a hitherto unknown soluble form of the enzyme or, alternatively, by residual microsomal contamination.

Table I shows the activities of four microsomal enzymes of the cholesterol biosynthetic pathway in an 3S100 preparation and in microsomes. Of the four tested enzymes, only HMG-CoA reductase is known to exist in a soluble truncated form with subunit size of 52-56 kDa as well as in a microsomal form with a subunit size of 97 kDa (Ness et al., 1981). Unlike the activity of squalene epoxidase and 2,3-oxidosqualene-lanosterol cyclase, which are strictly in the microsomes, the activity of squalene synthetase is observed in the S100 fraction as well as in the microsomes, as was also observed for HMG-CoA reductase. This difference in distribution of activities between the cyclase and epoxidase on one hand and squalene synthetase and HMG-CoA reductase on the other strongly
TABLE I
Activities of microsomal enzymes of the cholesterol biosynthetic pathway in microsomal and soluble preparations

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity</th>
<th>Microsomes</th>
<th>$S_{100}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol × mg$^{-1}$ × min$^{-1}$</td>
<td>pmol × min$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>HMG-CoA reductase</td>
<td>3800</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td>Squalene synthetase</td>
<td>2200</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>Squalene epoxidase</td>
<td>940</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>2,3-Oxidosqualene-lanosterol cyclase</td>
<td>240</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

TABLE II
Extraction of activity of squalene synthetase from microsomes by freeze-thaw

Crude $S_{100}$ preparation was obtained from livers of rats fed LO diet. The extract was centrifuged at 105,000 × g for 1 h, and the $S_{105}$ supernatant was removed. The microsomes were then frozen in liquid $N_{2}$, thawed, and once again centrifuged to obtain the first supernatant extract. This freeze-thaw centrifugation procedure was repeated two more times to obtain second and third $S_{105}$ extracts. The activity of squalene synthetase was determined in the original $S_{100}$ microsomal preparation as well as in the three freeze-thaw extracts.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Specific activity</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol × mg$^{-1}$ × min$^{-1}$</td>
<td>pmol × min$^{-1}$</td>
</tr>
<tr>
<td>$S_{100}$</td>
<td>0.3</td>
<td>210</td>
</tr>
<tr>
<td>First extract</td>
<td>4.3</td>
<td>360</td>
</tr>
<tr>
<td>Second extract</td>
<td>6.8</td>
<td>190</td>
</tr>
<tr>
<td>Third extract</td>
<td>1.8</td>
<td>47</td>
</tr>
</tbody>
</table>

indicates that the activity of squalene synthetase in the soluble extract is not due to the residual presence of microsomal particles in the $S_{100}$ preparation. Additional release of soluble squalene synthetase can be obtained by repeated freeze-thaw of the microsomes (Table II) similar to that described for HMG-CoA reductase (Heller and Gould, 1973; Edwards et al., 1980). Preparation of rat liver microsomes in the presence of protease inhibitors abolished the activity of cytosolic squalene synthetase and prevented its freeze-thaw extraction from the microsomes (data not shown).

Effect of Protease Inhibitors and Trypsin on the Size of Squalene Synthetase

We next attempted to release a solubilized form of squalene synthetase by protease treatment of microsomes. For this purpose, FL microsomes were isolated in the presence of the protease inhibitors described above and washed (three times) with buffer devoid of protease inhibitors. The release of solubilized squalene synthetase from these microsomes by trypsinization is shown in Fig. 5A. A time-dependent release of solubilized squalene synthetase was observed. Typically, 25–35% of the total microsomal activity was released in a solubilized form after 2 h of trypsinization under the conditions stated (Fig. 5A). Prolonged periods of exposure to trypsin beyond 2 h caused a decrease in the recovery of squalene synthetase activity. Similarly, increase of the temperature during proteolysis to 20 °C or higher resulted in loss of activity even at short time periods. The specific activity of the soluble enzyme recovered after 2 h of trypsinolysis is approximately 1–1.6 times higher than that in the original microsomal preparation (Fig. 5). Fig. 6A shows the elution of squalene synthetase activity of a crude enzyme preparation ($S_{100}$) that was prepared without protease inhibitors from a Sephacryl S-200 gel filtration column. As shown, there is no unique elution of activity, and the size distribution extends from the void volume to approximately 32 kDa. The presence of a mixture of protease inhibitors in the buffers, throughout the enzyme preparation procedure, resulted in a single peak of activity which was eluted at the void volume under the same chromatography condition (Fig. 6B). However, removal of the protease inhibitors by sequential washing of the microsomes followed by mild trypsinolysis as described above resulted in a soluble enzyme preparation (Fig. 6C) which eluted from the column under the same conditions at an estimated size of 32–36 kDa. Furthermore, this proteolysis product retained enzyme activity and showed remarkable stability in comparison to detergent-solubilized enzyme (data not shown). We concluded that this treatment led to proteolysis of squalene synthetase and the release of an enzymatically active fragment into solution. The above experiments served as a basis for the production of a truncated form of squalene synthetase which was then further purified.

Purification of Trypsin-solubilized Squalene Synthetase

The purification of trypsin-solubilized squalene synthetase was achieved through a series of standard column chromatography techniques employing ion-exchange and hydrophobic column chromatography followed by FPLC gel filtration on a Superose 12 column when necessary. Fig. 7A shows the first column chromatography purification step of the trypsin-solubilized enzyme on a DEAE-cellulose (DE52) ion-exchange column. Fractions 42–47, obtained from the DE52 column, were pooled and loaded onto a hydroxylapatite column. The
activity of squalene synthetase was eluted off the hydroxylapatite column with a salt gradient (Fig. 7B), and fractions containing the major peak of activity (fractions 54–58) were pooled. This enzyme was then loaded onto a phenyl-Sepharose column and eluted as described under “Experimental Procedures.” The purified enzyme was eluted at fractions 99–109 (Fig. 7C). The purified fractions were pooled and concentrated. This concentrated enzyme was either used directly for production of rabbit anti-sera or further chromatographed on gel filtration columns using Superose 12 FPLC chromatography. No further increase in specific activity was observed following the gel filtration chromatography of freshly isolated enzyme, and, therefore, it was used only to repurify aged enzyme. Using Superose 12 FPLC filtration chromatography for the estimation of the purified enzyme size indicated 35 kDa (data not shown) as was estimated by Sephacryl S-200 chromatography (see above). Fig. 8 shows an SDS-PAGE of the proteins obtained at the various purification steps.

Table III summarizes the purification and yields at the various purification steps. Typically, over 160-fold purification was achieved using the above procedure, and the purified enzyme was obtained at a yield of 5–10%. The enzyme migrates as a single peak on SDS-PAGE with an estimated size of 32–33 kDa (Fig. 8). Isoelectric focusing indicates a pI value of 5.6. The purified enzyme was characterized with respect to initial velocity kinetics. The $S_{0.5}$ for FPP was calculated to be 1.0 $\mu$M with $V_{\text{max}}$ of 1.2 $\mu$mol/min/mg. $S_{0.5}$ for NADPH was calculated to be 40 $\mu$M whereas NADH, which also served as

**Table III**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol × mg$^{-1}$ × min$^{-1}$</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Induced microsomes</td>
<td>7.7</td>
<td>100.0</td>
<td>1</td>
</tr>
<tr>
<td>Trypsin supernatant</td>
<td>8.7</td>
<td>25.0</td>
<td>1.1</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>38</td>
<td>12.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>250</td>
<td>5.9</td>
<td>33.8</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>1200</td>
<td>7.8</td>
<td>155.8</td>
</tr>
</tbody>
</table>
Purification of Rat Hepatic Squalene Synthetase

The purified enzyme was used for the preparation of anti-squalene synthetase monospecific rabbit antisera. Using this sera, we immunoanalyzed for squalene synthetase using Western blots. Fig. 9 shows such an analysis of the microsomal and trypsin-treated enzymes as well as the phenyl-Sepharose-purified enzyme. A single band was observed at 45–47 kDa for the microsomal enzyme and at about 32 kDa for the purified trypsin-treated enzyme. A smaller fragment (~28 kDa), which was removed during subsequent purification steps, was also observed with the trypsin-treated sample. Fig. 9 also illustrates the induction of enzyme mass in microsomes isolated from animals treated with fluvastatin when compared to animals maintained on a normal diet.

DISCUSSION

One important aspect that allowed the successful isolation and purification of the active truncated form of squalene synthetase is the development and implementation of a microassay in a 96-well plate that does not employ an organic extraction step. This allowed an efficient procedure for the analysis of large numbers of column fractions in a relatively short time. In addition, this assay permits the determination of squalene synthetase activity in tissue cultures without prior collection of the cells and extraction of enzyme. A key observation that allowed the development of a microassay was the activation of the enzyme by CHAPS and DTT. While DTT has limited effect on freshly isolated microsomes, CHAPS was found to be effective in the activation of both microsomal and solubilize enzyme (data not shown for the solubilized enzyme). The combination of both DTT and CHAPS in the assay proved to yield reproducible results in the determination of squalene synthetase activity in the various preparations.

Early attempts to solubilize yeast membrane squalene synthetase involved the use of detergent (Shechter and Bloch, 1971). The purification of the yeast-derived squalene synthetase was later achieved by employing nonionic detergents for the solubilization of the enzyme out of the membrane (Kuwik-Rabiega and Rilling, 1987; Sasiak and Rilling, 1988). Early attempts in our laboratory to isolate and purify the hepatic enzyme also involved the use of detergents. However, the observation that this enzyme, in the absence of protease inhibitors, showed partition of activity between the soluble and the microsomal fractions (Table I, Fig. 4) prompted us to consider its purification in the absence of detergents. While proteolysis of the yeast enzyme was observed and proved to be a problem for its purification (Sasiak and Rilling, 1988), we have taken advantage of this process for the isolation of an active truncated form of the enzyme. The evidence that shows the release of this enzyme in an active form to a soluble fraction, either at the early stage of preparation of the soluble extract (Table I, Fig. 4) or by the freeze-thaw procedure (Table II), does not indicate or prove the truncation of the protein to a smaller size enzyme. However, the reduction in protein size was assumed at that stage due to similar observations for HMG-CoA reductase which was shown to be released in a soluble truncated form from microsomes by endogenous proteolysis in the freeze-thaw procedure (Heller and Gould, 1972; Edwards, et al., 1980). This proteolysis can be inhibited by the presence of leupeptin in the preparation and preservation of the intact 97-kDa enzyme (Ness et al., 1981). Support for the possibility that hepatic squalene synthetase can also be proteolyzed out of the microsomes came first from preliminary studies in which freeze-thaw-treated microsomes were centrifuged in a sucrose gradient in the presence of 5 mM CHAPS. Activity was found in a protein of a smaller size (data not shown). However, quantitative inconsistencies prompted us to devise a better procedure for the proteolytic release of this enzyme. Employing mild trypsinolysis, we were able to release this enzyme out of the membrane in a soluble active form (Fig. 5). Additional similarities between squalene synthetase and HMG-CoA reductase were indicated by Ellenberg et al. (1989) that have shown that these two enzymes, unlike squalene epoxidase and 2,3-oxidosqualene-lanosterol cyclase, are not inhibited by digitonin. Since digitonin is known to interact with sterols in the membrane it was assumed that change in the membrane structure does not affect the catalytic site of either HMG-CoA reductase or squalene synthetase and brought out the possibility that the catalytic site of squalene synthetase is not integrated within the membrane.

Additional consideration for the hypothesis that squalene synthetase has a distinct cytosolic domain similar to HMG-CoA reductase comes from examination of the catalytic reaction itself. The substrates for HMG-CoA reductase are all water-soluble. The structural details of HMG-CoA reductase indicate that, although it is associated with and embedded in the ER, its catalytic site is located in an hydrophilic domain of the protein which protrudes into the cytosol. Thus, a release of a 52–56-kDa active truncated form of reductase is accomplished by proteolysis of this domain out of the membrane (Liscum et al., 1983, 1985). For squalene synthetase, all the substrates and metal ions required for the reaction (FPP, NADPH, Mg²⁺) are water soluble, and most of the products of the reaction (PP, NADP⁺) are water soluble as well. Thus, it is reasonable to assume that the catalytic site of this enzyme also protrudes into the cytosol to allow access of these substrates and removal of products. A strong indication that this is indeed the case comes from the release itself of an active form of the enzyme by mild proteolysis (Fig. 5) accompanied by a decrease in the size of the enzyme (Figs. 6 and 9). Since squalene is not water soluble, there still remains the question of its removal at each catalytic cycle. Under in vivo conditions,
there should be a mechanism for the removal of squalene and its transfer to the lumen of the membrane, therefore a carrier protein was considered earlier for this function (Shechter and Bloch, 1971). The removal of squalene in the assay is accomplished, most likely, by the presence of the detergent. This may explain the observed activation of the enzyme by CHAPS (Fig. 2).

The activity of squalene synthetase in fibroblasts is suppressed in the presence of low density lipoprotein (Faust et al., 1979). The presence of the bile acid sequestran cholerylamine in the diet of rats causes the increase in activity of hepatic squalene synthetase (Cohen et al., 1986). The results presented in Fig. 1 are in agreement with the reported data. As shown, cholesterol-fed rats show 60% suppression of squalene synthetase activity, whereas an increase of 11- and 25-fold in activity was observed for animals maintained on diets containing lovastatin and fluvastatin, respectively. While discussion on the significance of this observation in relationship to cholesterol homeostasis is beyond this article, this observation was nevertheless useful for the induction of hepatic squalene synthetase for the purpose of its isolation.

A point for further consideration should be the regulation of the turnover of the enzyme protein. For HMG-CoA reductase, it was shown that the degradation of the enzyme protein is under regulatory control (Edwards et al., 1983; Gill et al., 1985; Peffley and Sinensky, 1985; Naknashi et al., 1985; Skalnik et al., 1988), and the mechanism for the degradation involves the membrane-bound domain of the protein (Gill et al., 1985; Jingami et al., 1987; Skalnik et al., 1988). Unlike HMG-CoA reductase, the detailed structure of squalene synthetase in not yet known. However, the data presented in this work suggest the existence of both a membrane and a cytosolic domain for the squalene synthetase protein as well. Thus, it will be interesting in future studies to elucidate the degradation control of this enzyme and to investigate whether other similarities to HMG-CoA reductase exist.

Acknowledgments—We thank Dr. Larry Perez for preparation of the unlabeled farnesyl diphosphate. We also thank Dr. Faizulla Kathawala for useful discussions throughout these studies.

REFERENCES


Solubilization, purification, and characterization of a truncated form of rat hepatic squalene synthetase.
I Shechter, E Klinger, M L Rucker, R G Engstrom, J A Spirito, M A Islam, B R Boettcher and D B Weinstein


Access the most updated version of this article at http://www.jbc.org/content/267/12/8628

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/267/12/8628.full.html#ref-list-1