Studies on Squalene Epoxidase of Rat Liver*

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SUMMARY

Rat liver microsomes previously heated to 50° for 5 min accumulate 2,3-oxidosqualene on incubation with squalene. Squalene epoxidase activity can be assayed either with squalene and heated microsomes or with 10,11-dihydro-squalene and intact microsomes. In common with other monoxygenases, the epoxidase requires TPNH and molecular oxygen. Both a soluble fraction of rat liver and microsomes are necessary for enzyme activity. Carbon monoxide or potassium cyanide fail to inhibit squalene epoxidation.

The conversion in vitro of squalene to lanosterol was shown in this laboratory to be effected by the combined action of liver microsomes and soluble liver fraction and to require the presence of 1TPNH and oxygen (1). It was further shown that in the course of cyclization, atmospheric oxygen enters the substrate and provides the oxygen for the 3β hydroxyl group of the sterol (2).

The earlier view, holding that the oxidation and cyclization of squalene are fully concerted and proceed without intermediates to lanosterol (3), has lately been abandoned in favor of sequential oxidation and cyclization processes. This currently accepted two-step mechanism was proven independently in the laboratories of Corey and van Tamelen by the discovery of 2,3-oxidosqualene as an intermediate in the squalene-lanosterol transformation (4, 5). In support of the stepwise mechanism the isolation of a microsomal 2,3-oxidosqualene sterol cyclase has been described (6, 7). This enzyme does not require O2 and TPNH nor does it catalyze any transformation of squalene. By inference, therefore, a separate enzyme exists for converting squalene into 2,3-oxidosqualene. Indirect evidence for a squalene epoxidase has been obtained with the aid of the analogue 2,3-iminosqualene (8) and with 10,11-dihydrosqualene (9). The imino analogue is a potent cyclase inhibitor and causes the accumulation of 2,3-oxidosqualene in liver homogenates incubated with squalene. The 10,11 dihydro-derivative of squalene is epoxidized in the liver system but apparently not cyclized.

The present paper reports some properties of the squalene epoxidase system and describes a heat treatment of rat liver microsomes which abolishes cyclase activity without impairing the enzymatic conversion of squalene to 2,3-oxidosqualene.

EXPERIMENTAL PROCEDURE

Materials—14C-Squalene, prepared from DL-2,14C-mevalonic acid according to the method of Popjak et al. (10), 10,11-dihydrosqualene-13-H, synthesized by the method of Corey and Russey (9), 2,3-dihydrosqualene, 2,3-oxidosqualene (cold and 1-14C), and 2,3-iminosqualene were kindly supplied by Professor Corey and his associates of this department. 4,4'-Dimethyl-Δ7- and Δ8-(9)-3-ketocholestanediene were earlier synthesized in this laboratory by Dr. F. Gautschi.

Blood serum lipoprotein (Cohn’s Fraction IV-1) was supplied by the Blood Research Institute, Boston, Massachusetts. TPNH, TPN, DPNH, and DPN were purchased from Calbiochem. dl-Sodium isocitrate, sodium glucose 6-phosphate, isocitrate dehydrogenase type IV, and glucose 6-phosphate dehydrogenase type VI were obtained from Sigma. Silica gel for thin layer chromatography was obtained from Woelm, previously coated silica gel plates from Analtech, Inc., Wilmington, Delaware.

Enzyme Preparation—The suspending medium was 0.1 M potassium phosphate buffer, pH 7.5. Female rats (strain CD, body weight about 200 g) were killed by exsanguination. Livers were perfused with buffer, minced with scissors, and the minced tissue homogenized in a Potter-Elvehjem homogenizer with a loose-fitting Teflon pestle in the presence of 2 volumes of buffer.

The homogenate was centrifuged at 9,750 g for 10 min, and the supernatant was removed by decantation and then recentrifuged at 105,000 g for 60 min. The sediment (microsomes) was taken up in a volume of buffer equivalent to one-third the original liver weight. Active microsomes could also be prepared with 0.25 M sucrose or 1.15%; KC1 as suspending media.

Cyclase was prepared from hog liver microsomes as described (7).

Enzyme Assay—Epoxidase activity was assayed with 14C-squalene or 10,11-dihydrosqualene-13-H as substrates. The reaction mixture contained in 1 ml: potassium phosphate buffer, pH 7.5, 100 μmoles; TPNH, 1 μmole; ethylenediaminetetra-
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FIG. 1. Squalene conversion by intact and heated microsomes. 

$^{14}$C-Squalene was incubated for 60 min with microsomes and soluble fraction (17.9 mg of protein) under the standard conditions. Thin layer chromatography was carried out with the solvent system benzene-ethyl acetate (9:1). a, intact microsomes (4.2 mg of protein); b, microsomes (8.4 mg of protein) previously incubated at 50°C for 5 min and then incubated with squalene. S, squalene; O, 2,3-oxidosqualene; L, lanosterol; C, cholesterol.

FIG. 2. Squalene conversion by microsomes previously incubated at various temperatures. Microsomes (0.2 ml) were placed in Pyrex tubes (1-cm diameter) and kept for 5 min at the indicated bath temperature. Samples were then incubated with $^{14}$C-squalene under the standard condition. •--•, sterols; O--O, 2,3-oxidosqualene.

FIG. 3. Cyclase activity of heated microsomes. Intact and heated (50°C for 5 min) microsomes (33.4 mg of protein per ml) were mixed with half their volume of 3% sodium deoxycholate (7) and kept in ice for 15 min. Aliquots (0.1, 0.2, and 0.3 ml) were removed and their cyclase activity was determined with 2,3-oxidosqualene as substrate under the standard condition (7). O--O, intact microsomes; •--•, heated microsomes.

acetate, 1 mM, $^{14}$C-squalene, 70 μmol, 10,000 cpm; or 10,11-dihydrosqualene-13-3H, 40 μmol, 20,000 cpm; microsomes; and soluble fraction. A screw cap Pyrex culture tube (16 x 125 mm) containing the reaction mixture was incubated at 37°C for 30 min with shaking. The reaction was terminated by the addition of 1 ml of 10% methanolic potassium hydroxide. After 30 min, nonsaponifiable materials were extracted three times with 3 ml each of petroleum ether. Extracts were dried over anhydrous sodium sulfate and the solvent was evaporated under a nitrogen stream. The residue was dissolved in a small volume of ethyl ether and spotted on silica gel thin layer chromatography plates. The plates were developed to a height of 10 cm in methylene chloride-ethyl acetate (97:3). $R_f$ values were 0.87 for squalene or 10,11-dihydrosqualene, 0.55 for 2,3-oxidosqualene or 2,3-oxido-10,11-dihydrosqualene, 0.24 for lanosterol, and 0.13 for cholesterol. Plates were divided into three bands, squalene, epoxide, and sterol regions, and each band was scraped into a scintillation vial. Radioactivity was determined in toluene containing 0.4% 2,5-diphenyloxazole with a Packard liquid scintillation spectrometer, model 3003 (efficiency, 89% for $^{14}$C and 37% for $^3$H). Recoveries of radioactivity ranged from 70 to 90%.

Cyclase activity was assayed as described previously (6, 7). Aniline hydroxylase was measured by the method of Imai, Ito, and Sato (11). Protein was determined according to the method of Lowry et al. (12).

RESULTS

Epoxidation of 10,11-Dihydrosqualene

Corey and Russey have shown that 10,11-dihydrosqualene is epoxidized by liver homogenates at either of the two terminal double bonds of the hydrocarbon (9). The enzyme systems described here consisting of microsomes and supernatant also converted 10,11-dihydrosqualene-13-3H to the epoxide derivative as the main product as judged by thin layer chromatography. Depending on the epoxidase activity, a small but significant amount of unidentified radioactive material was distributed in more polar regions. 10,11-Dihydrosqualene was about half as active a substrate as squalene. For this reason and because labeled squalene is more conveniently prepared, squalene was the substrate in the majority of the experiments to be described.

$^1$ Since the properties of different lots of silica gel varied, the most effective solvent system was chosen in each case from the following: Solvent 1, benzene-ethyl acetate (93:1); Solvent 2, benzene-ethyl acetate (9:1); or Solvent 3, methylene chloride.
Identification of epoxidase product as 2,3-oxidosqualene by thin layer chromatography. Reaction mixtures contained potassium phosphate buffer, pH 7.5 (100 μmoles), TPNH (1 μmole), ethylenediaminetetraacetate (1 μmole), 14C-squalene (105 μmoles, 15,000 cpm), microsomes (8.8 mg of protein, previously incubated at 37° for 5 min), and soluble fraction (32.3 mg of protein) in a total volume of 1.5 ml. Five such tubes were incubated at 37° for 45 min. Nonsaponifiable compounds were isolated by standard methods and extracted into petroleum ether. Previously coated silicas gel plates were used for thin layer chromatography. One-tenth aliquot of the extract was chromatographed with methylene chloride as the developing solvent and the radioactivity of 0.5-cm sections measured (a). The remainder of the petroleum ether extract was subjected to thin layer chromatography in methylene chloride and the epoxide located with authentic 2,3-oxidosqualene as a marker which was visualized by iodine fumes. The epoxide region was scraped from the plate and extracted with ethyl ether. Two one-tenth portions of these ether extracts were rechromatographed separately in ethyl ether-n-hexane (3:7) (b), and in benzene (c). Another one-tenth aliquot was chromatographed in chloroform on a previously coated silver nitrate-silica gel plate (d). S, squalene; O, 2,3-oxidosqualene.

Conversion of epoxidase product to lanosterol by cyclase. a, reaction product was prepared on the same scale and isolated as described in Fig. 4. A three-tenths aliquot of the ethyl ether extract and 2.5 mg of nonradioactive 2,3-oxidosqualene were dissolved in 7 ml of dimethysothetane water (5:1), 5 drops of 70% perchloric acid were added, and the mixture was kept in an ice bath under nitrogen for 5 hours. The product was extracted with ethyl ether and chromatographed on a previously coated silicas gel plate with benzene-ethyl acetate (8:2) as developing solvent. b, a control experiment was carried out with racemic 2,3-oxidosqualene-l4C (2.7 mg, 11,000 cpm). L, lanosterol.

Acid treatment of epoxidase product. a, reaction product was prepared on the same scale and isolated as described in Fig. 4. A four-tenths aliquot of the ethyl ether extract and 2.5 mg of nonradioactive 2,3-oxidosqualene were dissolved in 7 ml of dimethysothetane water (5:1), 5 drops of 70% perchloric acid were added, and the mixture was kept in an ice bath under nitrogen for 5 hours. The product was extracted with ethyl ether and chromatographed on a previously coated silicas gel plate with benzene-ethyl ether (8:2) as developing solvent. b, a control experiment was carried out with racemic 2,3-oxidosqualene-l4C (2.7 mg, 11,000 cpm). DS, 2,3-dihydroxyoxidosqualene.

<table>
<thead>
<tr>
<th>System</th>
<th>2,3-</th>
<th>2,3-Oxidosqualene</th>
<th>2,3-Oxido-10,11-dihydroxyosqualene</th>
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<tr>
<td>O2</td>
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* For unknown reasons, the recovery of radioactivity was poor (about 50%) when the soluble fraction was incubated with 14C-squalene or 14H-10,11-dihydroxyosqualene in the absence of microsomes and the saponified reaction mixture extracted with petroleum ether. The radioactive compound remaining in the reaction mixture could be extracted with ethyl acetate and was identified as squalene by thin layer chromatography and gas chromatography. The values given are appropriately corrected.

Cyclase Inactivation by Heat Treatment of Microsomes

When microsomes and the soluble fraction were incubated with 14C-squalene in the presence of TPNH and air and the products analyzed by thin layer chromatography, most of the radioactivity, apart from that in squalene, was found in the sterol region (Fig. 1a). Little epoxide accumulated under these conditions. Prior heating of the microsomes markedly changed this pattern. Microsomes previously treated for 5
min at 45° were still able to catalyze the conversion of squalene to sterols (Fig. 2). However, preliminary treatment at 50° for 5 min destroyed this ability and the reaction product was now almost entirely localized in the area corresponding to 2,3-oxidosqualene (Figs. 1b and 2). Microsomal cyclase is thus markedly more heat-sensitive than epoxidase and by virtually eliminating it one can arrest squalene metabolism at the epoxide stage (Fig. 3).

The reaction product obtained with heat-treated microsomes was indistinguishable from 2,3-oxidosqualene on thin layer chromatography with several solvent systems (Fig. 4) and moreover, it was converted quantitatively to lanosterol when incubated with cyclase (Fig. 5). Treatment with perchloric acid to cleave the epoxide ring yielded a more polar product which over, it was converted quantitatively to lanosterol when incubated at 50° for 5 min, and soluble fraction as indicated. Incubation was carried out at 37° for 30 min.

Requirements for Squalene Epoxidase

The components required for epoxidase activity are shown in Table I. They include the soluble (supernatant) liver fraction in addition to TPNH, oxygen, and microsomes. TPNH at 1 mM was sufficient to sustain a linear reaction rate for 30 min under standard conditions. TPNH at 1 mM was sufficient to sustain a linear reaction rate for 30 min under standard conditions. The addition of glucose 6-phosphate dehydrogenase or isocitrate dehydrogenase TPNH-generating systems. 

As shown in Fig. 7, epoxidase activity is completely dependent on the soluble fraction which is needed in relatively large amounts. This requirement also holds when microsomes are prepared in 0.25 M sucrose or 1.15% KCl. The soluble fraction could not be replaced by the glucose 6-phosphate dehydrogenase or isocitrate dehydrogenase TPNH-generating systems.

In preliminary experiments the following properties of the supernatant factor or factors were noted. When kept in 0.1 M potassium phosphate buffer, pH 7.5, and heated for 5 min, the soluble fraction lost activity at temperatures above 50°. Acetone precipitation (80%) gave good recovery and ammonium sulfate precipitation (60% saturation) poor recovery of activity in the precipitates.

On dialysis against 100 volumes of 0.1 M potassium phosphate buffer, pH 7.5, activity declined to about one-third of the original after 12 to 20 hours. Activity of the soluble fraction was partially lost when it was passed through a Sephadex G-25 column equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, but not totally. Boiled (2 min) soluble fraction restored the dialyzed soluble fraction to almost full activity, but this effect was not always reproducible. The soluble fraction was also applied to a Sephadex G-200 column equilibrated with 0.1 M potassium phosphate buffer at pH 7.5 and the activity of each fraction assayed under standard conditions in the presence of boiled supernatant. The active fractions appeared to be somewhat less retarded than ovalbumin (molecular weight, 45,000).

The above results suggest that the supernatant fraction contributes both a protein and a heat-stable low molecular weight factor as essential components of epoxidase activity.
Effect of Inhibitors

Carbon Monoxide—Epoxidase and aniline hydroxylase as a control were assayed in a gas phase containing carbon monoxide—oxygen (95:5). While aniline hydroxylase was markedly inhibited under these conditions, squalene epoxidase was not affected (Table II). In nitrogen—oxygen (95:5), epoxidase activity was 60 to 80% of that in air. The resistance of epoxidase to CO has been observed independently.2

Metal-chelating Agent—The following reagents (at 1 mm) had no significant inhibitory effect on epoxidase: KCN, NaCN, NH₂OH, EDTA, or γ-phenanthroline.

Squalene Derivatives and Fatty Acids—2,3-Oxidosqualene, the reaction product (80 μm as the racemic mixture), and 2,3-iminosqualene (0.01 to 0.1 mm) were without effect on epoxidase. The following unsaturated fatty acids (as sodium salts) were potent inhibitors at 0.2 mm: steardolate (100% inhibition), oleate (90%), linoleate (94%), and linolenate (96%). Stearate was not inhibitory.

Lipoprotein—Serum prepared from rat blood reduced epoxidase activity as follows: 0.05 ml of serum per ml of reaction mixture, 24%; 0.10 ml, 35%; 0.20 ml, 53%; and 0.30 ml, 63%. The inhibition persisted after boiling the serum for 2 min. Serum lipoprotein (Cohn's Fraction IV-1) likewise inhibited epoxidase activity in all preparations tested, information on its function is lacking. Since epoxidase is an enzyme that acts on unsaturated compounds, it is conceivable that the inhibition is due to the presence of saturated fatty acids in the lipoprotein fraction and a heat-stable small molecule. Satisfactory procedures for the separation of these factors remain to be worked out.

Metallooxynases containing the cytochrome P-450 as a prosthetic group are inhibited by carbon monoxide (17). Liver stearyl-CoA desaturase, another enzyme depending on O₂ and TPNH, is cyanide-sensitive (18) presumably because a metalloenzyme is involved. Neither carbon monoxide nor cyanide inhibits squalene epoxidase. The nature of the electron carrier system in the squalene epoxidation remains, therefore, undetermined.

Lipoprotein causes squalene accumulation in liver homogenates incubated with mevalonate. The inhibition of squalene epoxidase by serum or lipoprotein (Cohn's Fraction IV-1) is consistent with this observation.

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REFERENCES

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