Purification of a 3β-Hydroxy-Δ5-C27-sterol Dehydrogenase from Pig Liver Microsomes Active in Major and Alternative Pathways of Bile Acid Biosynthesis*

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A 3β-hydroxy-Δ5-C27-sterol dehydrogenase active in bile acid biosynthesis was purified from pig liver microsomes by solubilization with sodium cholate and by chromatography on DEAE-Sepharose, aminoethyl-Sepharose, and blue Sepharose. The last step in the purification procedure was preparative isoelectric focusing in a Rotofor cell. The final enzyme preparation showed only one protein band upon SDS-polyacrylamide gel electrophoresis. The isoelectric point was estimated to about 7.0 and the apparent M, was 36,000.

The purified enzyme catalyzed the conversion of 7α-hydroxycholesterol, 7α,25-dihydroxycholesterol, 7α,27-dihydroxycholesterol, and 3β,7α-dihydroxy-5-cholestenoic acid into the corresponding 3-oxo-Δ4 compounds. The enzyme was inactive with C19 and C21 sterols as substrates. The enzyme was also inactive with C27 sterols having the 7-hydroxy group in α instead of β-position. The Km was found to be 0.30 and 0.32 μM with 7α-hydroxycholesterol and 7α,27-dihydroxycholesterol as substrates, respectively. NAD+ was the preferred cofactor. A monoclonal antibody raised against the 3β-hydroxy-Δ5-C27-sterol dehydrogenase was prepared. After coupling to Sepharose, the antibody was able to bind the dehydrogenase and to decrease the conversion of 7α-hydroxycholesterol into 7α-hydroxy-4-cholest-3-one by more than 90%. The N-terminal amino acid sequence was determined and found to be similar but not identical with those of known 3β-hydroxy-Δ5-sterol dehydrogenases active in steroid hormone biosynthesis. Thus, the purified enzyme active toward C27 sterols in bile acid biosynthesis appears to represent a novel type of 3β-hydroxy-Δ5-sterol dehydrogenase.

In the major pathways of bile acid biosynthesis, the initial 7α-hydroxylation of cholesterol is followed by oxidation of the 3β-hydroxy group and isomerization of the Δ5 double bond (1). The oxidation and the isomerization are believed to be catalyzed by a single enzyme (2, 3). Other pathways to primary bile acids, not involving 7α-hydroxylation of cholesterol, have been described (4–6). It was shown recently that in the pig and in man 7α-hydroxylation of 27-oxygenated sterols is catalyzed by enzymes different from the cholesterol 7α-hydroxylase (7–9). It is not known whether the oxidation at C-3 of the side chain oxygenated steroids and 7α-hydroxycholesterol is catalyzed by the same enzyme.

In 1981, Wikvall (3) purified a 3β-hydroxy-Δ5-C27-sterol dehydrogenase from rabbit liver microsomes. This enzyme was active toward 7α-hydroxycholesterol. Side chain oxygenated steroids were not tested as substrates for the enzyme (3). In rabbit, the extent of 7α-hydroxylation of 27-oxygenated intermediates is very low. Consequently, alternative pathways involving 27-oxygenated intermediates may not be important in rabbit. The aim of the present study was to purify and characterize pig liver microsomal 3β-hydroxy-Δ5-C27-sterol dehydrogenase(s) active in bile acid biosynthesis.

**EXPERIMENTAL PROCEDURES**

Materials—[4,14C]Cholesterol was from Amersham Corp. (Buckinghamshire, UK). 7α-Hydroxycholesterol was from Steraloids Inc. (Wilton, NH). 25-Hydroxycholesterol, pregnenolone, and dehydroepiandrosterone were from Steraloids Inc. and used as starting material for synthesis 7α,25-dihydroxycholesterol (5-cholestane-3β,7α,25-triol), 7α-hydroxy-5-cholestene-3β,7α-dihydroxycholesterol, and 7α-hydroxydehydroandrosterone respectively (10). 27-Hydroxycholesterol was prepared from kryptogen in kindly supplied by Dr. L. Tökes, Syntex Research (Palo Alto, CA) and used for synthesis of 7α,27-dihydroxycholesterol (5-cholestene-3β,7α,27-triol), 7β,27-dihydroxycholesterol (5-cholestene-3β,7β,27-triol), 3β,7α-dihydroxy-5-cholestenolic acid, and 3β,7α-dihydroxy-5-cholestenolic acid (10). Chromatographic column packings were from Pharmacia Biotech Inc., Uppsala, Sweden.

Enzyme Purification—Liver microsomes were prepared from castrated, otherwise untreated, 6-month-old male pigs. The microsomes were suspended in 100 mM Tris-HCl buffer, pH 7.5, containing 20% (v/v) glycerol and 0.1 mM EDTA, solubilized with 1.8% (w/v) sodium cholate (6 mg protein/ml) and precipitated with polyethylene glycol (11). The proteins precipitating between 14 and 22% (w/v) polyethylene glycol (6000) were collected by centrifugation and dissolved in 10 mM phosphate buffer, pH 7.6, containing 0.4% (w/v) polyoxyethylene(10)lauryl ether (POELE). The buffer also contained 20% (v/v) glycerol and 0.1 mM EDTA as did all other buffers used in the purification procedure. Phosphate buffers were used as the potassium salt. The dissolved precipitate was subjected to chromatography on DEAE-Sepharose (5 x 30 cm) equilibrated in 10 mM phosphate buffer, pH 7.6, containing 0.4% POELE. The column was washed with the equilibrating buffer and then eluted with a linear gradient of 40–120 mM phosphate buffer, pH 7.6, containing 0.4% POELE. Fractions containing activity toward 7α-hydroxycholesterol were pooled and concentrated using a Minipart unit (Millipore) with four 10 kDa membranes. The concentrated sample was dialyzed against 10 mM phosphate buffer, pH 7.6, containing 0.2% POELE and 0.5% sodium cholate and applied to a second DEAE-Sepharose column (3 x 30 cm) equilibrated in the same buffer. The column was eluted with 35 mM phosphate buffer, pH 7.6, containing 0.2% POELE and 0.5% sodium cholate. The fractions containing enzyme activity were pooled and non-ionic detergent was removed by treatment with Amberlite XAD-2 (12) prior to dilution with five volumes of 100 mM phosphate buffer, pH 7.4, containing 0.5% sodium

* The abbreviations used are: POELE, polyoxyethylene(10)lauryl ether; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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cholate. The fraction was applied to an aminohexyl-Sepharose column (3 x 10 cm) equilibrated in the same buffer containing 0.7% sodium cholate. The column was washed with the equilibrating buffer and protein was eluted with 100 mM phosphate buffer, pH 7.4, containing 0.3% sodium cholate and 0.1% POELE. Fractions containing enzyme activity were pooled and concentrated using a Diaflo ultrafiltrating unit (Amicon) with a PM 10 filter and then dialyzed against 10 mM phosphate buffer, pH 7.4, containing 0.4% POELE. The dialysate was subjected to chromatography on a blue Sepharose column (2 x 10 cm) equilibrated in the dialysis buffer. The column was washed with the equilibrating buffer and then with the same buffer containing 60 mM KCl. Enzyme activity was eluted with the equilibrating buffer containing 300 mM KCl. The fractions containing enzyme activity were collected, concentrated with a PM 10 filter and dialyzed against 10 mM phosphate buffer, pH 7.0, containing 0.5% POELE. The dialysate was subjected to preparative isoelectric focusing using a Rotofor unit (Bio-Rad), prefocused for one hour with preblended ampholines ranging from pH 5.0 to 8.0 in 20% glycerol and 0.5% POELE. After 4 h of focusing, the fractions were collected and assayed for enzyme activity. Purity of each active fraction was tested by SDS-polyacrylamide gel electrophoresis and enzymatically active fractions showing a single protein band were pooled and dialyzed against 10 mM phosphate buffer, pH 7.4. The dialyzed preparation was used for enzymatic studies. The purification procedure is summarized in Fig. 1.

Protein was determined by the biuret reaction enhanced with bicinchoninic acid (BCA). When appropriate, the protein was first precipitated with trichloroacetic acid and sodium deoxycholate (14).

Production and Purification of Monoclonal Antibodies—An 8-week-old female mouse of the Balb/c strain was used. Purified 3β-hydroxy-Δ5,27-steroid dehydrogenase was emulsified with an equal volume of Freund's complete adjuvant. Fifty μg of the enzyme was injected intraperitoneally. Five weeks later, another 100 μg of the same antigen in 0.9% NaCl were injected intraperitoneally. Three days later the mouse was killed and the spleen was used as source of antibody-producing cells. The spleen cells were mixed 5:1 with Sp2/0 myeloma cells and fused using 50% polyethylene glycol. The hybridoma cells were incubated with microsomes (1 mg of protein) or purified enzyme (0.2 μg of protein) and 1 mg of NAD+ in a total volume of 1 ml of 100 mM phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. Incubations with 3β,7α-hydroxycholesterol (filled circles, solid line), 7α,25-dihydroxycholesterol (dotted line), and 7α-hydroxypregn-5-en-3-one were terminated by addition of 3 ml of methanol and kept at -20°C until analyzed. All other incubations were terminated with 5 ml of trichloroacethanol and (21).

Analysis of Incubation Mixtures—Incubations with 3β,7α-dihydroxy-5-cholesten-3-one, 3β,7β-dihydroxy-5-cholesten-3-one, 7α,25-dihydroxycholesterol, 7α,27-dihydroxycholesterol, 7α-hydroxypregn-5-en-3-one, and 7α-hydroxydehydroepiandrosterone were analyzed by gas liquid chromatography using a Shimadzu gas chromatograph equipped with a flame ionization detector and a Carbowax 20M capillary column (15 m x 0.25 mm). The column was heated at a rate of 3°C/min from 100°C to 240°C. Hydrogen was used as carrier gas at a flow rate of 30 ml/min. The detection limit was 0.1 nmol for each compound.

Table I. Purification of 3β-hydroxy-Δ5,27-steroid dehydrogenase from pig liver microsomes

| Fraction | Protein | Product formed | Yield | Purification |
|----------|---------|----------------|-------|-------------|
| Microsomes | 60,000 | 1.9 | 100% | 1 |
| Polyethylene glycol | 11,600 | 10.8 | 110% | 5.7 |
| DEAE-Sepharose | 282 | 92 | 23% | 48 |
| Aminohexyl-Sepharose | 56 | 352 | 17% | 185 |
| Blue-Sepharose | 8 | 1,790 | 13% | 942 |
| Isoelectric focusing | 0.5 | 2,880 | 1.3% | 1515 |

Fig. 1. General scheme for purification of 3β-hydroxy-Δ5,27-steroid dehydrogenase from pig liver microsomes.

Fig. 2. Chromatography on preparative isoelectric focusing. Elution of protein (open circles, solid line, dehydrogenase activity toward 7α-hydroxycholesterol (filled circles, solid line), pH (open circles, dotted line).

Fig. 3. SDS-polyacrylamide gel electrophoresis of collected fractions from the preparative isoelectric focusing. Fractions 11 to 16 were pooled and used for enzymatic studies and N-terminal amino acid sequence analysis.
chromatography-mass spectrometry as described previously (8).

Incubations with [14C]pregnenolone, [14C]dehydroepiandrosterone, and [14C]cholesterol were analyzed by thin layer chromatography and radioactive scanning as described previously (16).

To incubations with 7α-hydroxycholesterol, testosterone was added as an internal recovery standard. The organic phase was evaporated under nitrogen, dissolved in mobile phase, and subjected to high performance liquid chromatography (HPLC) on a silica column (LiChrosorb, 150 mm) with hexane/isopropanol (96:4) as mobile phase. Compounds with 3-oxo-D4 structure were monitored at 240 nm. Peak areas of product and internal standard were measured and the conversions were calculated from a standard curve. The retention times were 6.2 min for 7α-hydroxy-4-cholesten-3-one and 12.2 min for testosterone. The standard curve was prepared using known amounts of 7α-hydroxy-4-cholesten-3-one and was linear in the part used for calculation of conversions. The testosterone used as internal standard was purified on HPLC in the same system as was used for the incubations.

To incubations with 7α,27-dihydroxycholesterol, 11β-hydroxyprogesterone was added as an internal recovery standard. The organic phase was collected, evaporated under nitrogen and dissolved in mobile phase. The samples were subjected to HPLC on a silica column (LiChrosorb, 150 mm) with hexane/isopropanol (92:8) as mobile phase. Compounds with 3-oxo-D4 structure were monitored at 240 nm. Peak areas of product and internal standard were measured and the conversions were calculated from a standard curve. The retention times were 9.8 min for 7α,27-dihydroxy-4-cholesten-3-one and 13.2 min for 11β-hydroxyprogesterone. The standard curve was prepared using known amounts of 7α,27-dihydroxy-4-cholesten-3-one and was linear in the part used for calculation of conversions. The 11β-hydroxyprogesterone used as internal standard was purified on HPLC in the same system as was used for the incubations.

Incubations with 27-hydroxycholesterol were analyzed in a similar system as the two described above for 7α-hydroxycholesterol and 7α,27-dihydroxycholesterol. The mobile phase was hexane/isopropanol (98:2)
and the retention time was 11.6 for 27-hydroxy-4-cholesten-3-one. Androstenedione was used as internal recovery standard and had a retention time of 15.1 min.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (17) with 11% polyacrylamide (11% acrylamide, 2.9% bisacrylamide) gels, containing 0.1% (w/v) SDS. The samples were pretreated with SDS and β-mercaptoethanol at 100 °C for 1 min. The gels were stained as described by Wray et al. (18) or used for immunoblotting.

Incubations with Antibody-coupled Sepharose—Incubations with 3β-hydroxy-Δ5-C27-steroid dehydrogenase and Sepharose-bound monoclonal antibody (mAb 4F5) were performed as follows.

Thirty pmol of the dehydrogenase were incubated with 10, 25, and 50 μg of the antibody-coupled Sepharose in 300 μl of 100 mM PO4, pH 7.4, 20% glycerol, 0.1 mM EDTA, and 0.1% CHAPS. An irrelevant Sepharose-bound monoclonal antibody raised against mitochondrial CYP27 from pig liver and Sepharose without antibody were used as controls. After incubation in a rotating mixer for 1 h at room temperature followed centrifugation (4000 × g, 10 min, 4 °C) the Sepharose was washed twice with 200 μl of 100 mM PO4, pH 7.4, 20% glycerol, 0.1 mM EDTA, and 0.1% CHAPS and pooled. The supernatants were used for assay of remaining enzyme activity. The reaction mixtures were incubated with 20 μg of 7α-hydroxycholesterol for 10 min at 37 °C. The incubations were terminated, extracted and analyzed as described under "Incubation Procedure."

Amino-terminal Amino Acid Sequence Analysis—Purified enzyme, about 100 pmol, was subjected to SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride-membrane (Bio-Rad) as described by Towbin et al. (19), Matsudaira (20), and Moos et al. (21). The membrane was stained with 0.025% (w/v) of Coomassie Brilliant Blue R-250 in 40% (v/v) methanol and destained in 50% methanol. The protein bands were cut out and N-terminal amino acid sequence was determined as described previously (22) in a Applied Biosystems 473A sequencer.

Calculation of Kₘ Values—The calculation of the Kₘ values with 7α-hydroxycholesterol and 7α,27-dihydroxycholesterol were carried out with linear regression analysis.

### RESULTS AND DISCUSSION

Purification of 3β-Hydroxy-Δ5-C27-steroid Dehydrogenase—The purification of 3β-hydroxy-Δ5-C27-steroid dehydrogenase from pig liver microsomes is summarized in Table I. After two DEAE-Sepharose chromatography steps, all heme-containning proteins were completely removed. The preparation at this stage was slightly yellow, which was still the case after amionoehxyl-Sepharose chromatography. Most of the activity was bound to the blue Sepharose, as is common for many NAD⁺- and NADP⁺-dependent dehydrogenases.

The large loss of activity in some of the chromatographic steps can only be partly explained by activity in adjacent fractions. Inactivation of enzyme molecules during the purification may be substantial. In support of this it was found that when purified enzyme was preincubated with buffer without glycerol for 10 min at 37 °C, only 10% of the activity was left compared with enzyme preincubated with buffer containing 20% glycerol. In and after, the last step in the purification, the preparative isoelectric focusing (Fig. 2), the loss of activity was considerable. The focusing process and/or components in the buffer, e.g. nonionic detergent in very high concentration that can not be dialyzed, could be possible explanations for this loss.

The final, colorless, preparation was purified at least 1,500-fold as compared with the original microsomal fraction, although the above mentioned activity loss indicates an even higher degree of purification. The preparation showed only one protein band upon SDS-polyacrylamide gel electrophoresis, and the apparent Mᵣ was 36,000 (Fig. 3). The fact that the preparation is colorless argues against a possible flavin component. This is in line with findings of Wikvall (3). The apparent Mᵣ of the 3β-hydroxy-Δ5-C27-steroid dehydrogenase purified from pig liver microsomes in the present communication was much lower than that of the corresponding enzyme (Mᵣ = 46,000) purified from rabbit liver microsomes (3). Immunological and structural comparisons are not possible to carry out since attempts to characterize the rabbit liver enzyme further...

### Table II

| Substrate                  | Conversion (nmol/ (mg x min)) |
|----------------------------|------------------------------|
| Cholesterol                | 1489                         |
| 7α-Hydroxycholesterol      | 33                           |
| 27-Hydroxycholesterol      | 838                          |
| 7α,25-Dihydroxycholesterol | 175                          |
| 7α,27-Dihydroxycholesterol | 14                           |
| 3β,7α-Dihydroxy-5-cholestenolic acid | —  |
| 3β,7α-Dihydroxy-5-cholestenolic acid | —  |
| Pregnenolone               | —                            |
| 7α-Hydroxy-pregnenolone    | —                            |
| Dehydroepiandrosterone     | —                            |
| 7α-Hydroxydehydroepiandrosterone | —  |

*No detectable activity.*
Liver Microsomal 3β-Hydroxy-Δ5,7-C27-steroid Dehydrogenase

Comparison of the N-terminal amino acid sequence of the pig liver 3β-hydroxy-Δ5,7-C27-steroid dehydrogenase with the N-terminal amino acid sequences of human, macaque, bovine, rat and mouse 3β-hydroxy-Δ5,7-steroid dehydrogenases (23)

| Species   | Sequence                                           |
|-----------|----------------------------------------------------|
| Pig liver | AQQXLYLVQAGGFLGER                                  |
| Human I   | MTGWSCLTVGAGGFLQQR                                 |
| Human II  | MGWSCLTVGAGGLLQQR                                  |
| Macaque   | MTGWSCLTVGAGGFLQQR                                 |
| Bovine    | MAGWSCLTVGAGGFLQQR                                 |
| Rat I     | MPWSCLTVGAGGFGQQR                                  |
| Rat II    | MPWSCLTVGAGGGVQR                                   |
| Rat III   | MPWSCLTVGAGGGVQR                                   |
| Mouse I   | MAGWSCLTVGAGGFLQQR                                 |
| Mouse III | MGWSCLTVGAGGFLQQR                                  |

have not been successful.2

Assay Conditions and Substrate Specificity of Purified 3β-
Hydroxy-Δ5,7-C27-steroid Dehydrogenase—Assay conditions were determined with 7α-hydroxycholesterol as substrate (Fig. 4A–E). Only one product was detected, 7α-hydroxy-4-cholesten-3-one. NAD was the preferred cofactor and the enzyme was saturated with about 0.2 mM NAD+. NAD was only slightly active in the oxidation. The rate of oxidation was almost linear up to 10 min and 0.5 μg of enzyme. The pH optimum was around 7.5. The purified enzyme catalyzed the conversion, in order of efficiency, of 7α-hydroxycholesterol, 7α,27-dihydroxycholesterol, 3β,7α-hydroxy-5-cholestenolic acid, and 7α,25-dihydroxycholesterol into the corresponding 3-oxo-Δ4 compounds (Table I). The apparent K for 7α-hydroxycholesterol as substrate was 0.30 μM and for 7α,27-dihydroxycholesterol as substrate 0.32 μM. Calculated Vmax values in these experiments were low compared to the activity obtained directly after preparative isoelectric focusing. The K for 7α-hydroxycholesterol was considerably lower and the Vmax considerably higher than those reported previously for the rabbit liver enzyme (3).

Cholesterol, 27-hydroxycholesterol, 7β,27-dihydroxycholesterol, 3β,7β-dihydroxy-5-cholestenolic acid, pregnenolone, 7α-hydroxy pregnenolone, dehydroepiandrosterone, and 7α-hydroxy dehydroepiandrosterone were not oxidized by the enzyme. Thus, only C27 steroids with a hydroxy group in 7α-position are substrates for this 3β-hydroxy-Δ5,7-steroid dehydrogenase.

Immunological Studies—A monoclonal antibody raised against the pig liver microsomal 3β-hydroxy-Δ5,7-steroid dehydrogenase was prepared by immunization of a mouse with the purified enzyme. The antibody designated mAb 4F5 recognized the purified enzyme as well as a protein of the same apparent molecular weight as the enzyme (Fig. 5). The antibody was coupled to Sepharose and incubated with 3β-hydroxy-Δ5,7-steroid dehydrogenase. After incubation, the antibody-coupled Sepharose was removed and the supernatant was assayed for dehydrogenase activity. The conversion of 7α-hydroxycholesterol into 7α-hydroxy-4-cholesten-3-one was studied. Sepharose without antibody and Sepharose coupled to an irrelevant antibody directed against mitochondrial CYP27 from pig liver were used as controls. Fig. 6 shows that mAb 4F5, but not the irrelevant antibody, was able to bind the dehydrogenase and immunoprecipitate the enzymatic activity by more than 90%. These results confirm that the 36,000 M protein is the 3β-hydroxy-Δ5,7-C27-steroid dehydrogenase.

Structural Characterization—The N-terminal amino acid se-

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