HPLC purification and preparation of antibodies to cholic acid-inducible polypeptides from *Eubacterium* sp. V.P.I. 12708

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Abstract The role of bile acid-inducible polypeptides in 7-dehydroxylation was investigated in *Eubacterium* sp. V.P.I. 12708. Cholic acid-inducible bile acid 7α-, 7β-dehydroxylase, and Δ^6 reductase activities co-eluted from a gel filtration high performance liquid chromatography (HPLC) column. Antibody (Ab) was prepared to these enzymatically active fractions, immunoadsorbed with uninduced cell extract coupled to Sepharose 4B, and used for immunoprecipitation of [^{35}S]-methionine-labeled polypeptides. Ab immunoprecipitated polypeptides with molecular weights of 45,000, 27,000, and 23,500 from induced but not uninduced cell extracts. Immunoinhibition experiments showed that this Ab preparation inhibited (60%) bile acid 7α-dehydroxylase activity in cell extracts. The 45,000 mol wt polypeptide was purified by $(NH_4)_2SO_4$ fractionation, HPLC gel filtration, and HPLC–DEAE chromatography. Ab prepared to the 45,000 mol wt polypeptide immunoprecipitated only that polypeptide. This Ab, however, did not inhibit bile acid 7α-dehydroxylase activity. Ab specific for the 27,000 mol wt polypeptide was prepared by partial purification and immunoadsorption with uninduced cell extracts. Immunochemical staining, following SDS-PAGE of crude cell extracts, shows a single immunoreactive protein band at 27,000 daltons. This Ab immunoprecipitated the 27,000 mol wt polypeptide as well as small amounts of the 45,000 and 23,000 mol wt polypeptides. Immunoinhibition studies showed that this Ab preparation inhibited (25%) 7α-dehydroxylase activity. These data suggest that the 27,000 mol wt polypeptide is involved in enzyme catalysis. This does not, however, eliminate some role for the 45,000 and 23,500 mol wt polypeptides in bile acid metabolism in this organism.—**Paone, D. A. M., and P. B. Hylemon.** HPLC purification and preparation of antibodies to cholic acid-inducible polypeptides from *Eubacterium* sp. V.P.I. 12708. *J. Lipid Res.* 1984. **25:** 1343–1349.

Supplementary key words bile acid 7-dehydroxylase • HPLC-chromatography • antibody preparation

The 7α-dehydroxylation of cholic acid and chenodeoxycholic acid results in the formation of deoxycholic acid and lithocholic acid, respectively. The mechanism of this reaction, as proposed by Samuelsson (1) occurs by the diaxial trans-elimination of the 7α-hydroxyl group and 6β-hydrogen atom to form a Δ^6-intermediate. This compound is subsequently reduced to form the secondary bile acids.

Eubacterium sp. V.P.I. 12708 has an inducible (2) bile acid 7-dehydroxylase. Extracts from this bacterium have been shown to directly 7β-dehydroxylate ursodeoxycholic acid (3) and to reduce the Δ^6-intermediate (3α-hydroxy-5β-6-cholen-24-oic acid) to lithocholic acid (4, 5). Moreover, 7α-, and 7β-dehydroxylation activities and the reduction of the Δ^6-intermediate are all induced by cholic acid, stimulated by NAD^+, and inhibited by NADH (5). Analysis of HPLC gel filtration column fractions showed that the 7α-, 7β-dehydroxylation and Δ^6 reductase activities all co-eluted in a single peak (5). These observations suggest, but do not prove, that all three activities may be catalyzed by the same enzyme.

The induction of bile acid 7-dehydroxylase activity by cholic acid is accompanied by the appearance in whole cells of at least five new polypeptides (4) with molecular weights of 77,000, 56,000 (two polypeptides), 27,000, and 23,500. It is still unclear, however, which of these polypeptides constitute bile acid 7-dehydroxylase. Moreover, attempts to purify an enzymatically active bile acid 7-dehydroxylase by conventional methods have been unsuccessful due to irreversible inactivation of this enzyme. In this communication, we report the preparation of antibodies to bile acid 7-dehydroxylase and the purification of bile acid-inducible polypeptides using HPLC techniques.

MATERIALS AND METHODS

Culture of bacteria and preparation of cell extracts

Characteristics, growth conditions, and media composition for the anaerobic culturing of *Eubacterium* sp.

Abbreviations: Systematic names of bile acids referred to in the text by their trivial names are as follows: cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholan-24-oic acid; lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid, Δ^6-intermediate, 3α-hydroxy-5β-6-cholen-24-oic acid; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; V.P.I. Virginia Polytechnic Institute.

V.P.I. 12708 has been described previously (2). Bacterial cultures, in 3-liter volumes, were induced to synthesize bile acid 7-dehydroxylase by the addition of sodium cholate (0.1 mM) at hourly intervals during logarithmic growth. Cell extracts were prepared anaerobically as described previously (4). Protein concentration of cell extracts was determined by the procedure of Kalb and Bernlohr (6).

Assay for bile acid dehydroxylase

Enzymatic 7α-dehydroxylation of [24-^{14}C]cholic acid was followed by measuring the rate of [24-^{14}C]deoxycholic acid formation using a radiochromatographic assay as described previously (7). The standard assay mixture contained in a final volume of 0.5 ml: 25 mM sodium acetate-morpholinopropane sulfonate (MOPS, pH 7.5); 100 μM cholic acid (containing 0.1 μCi of [24-^{14}C]cholic acid); 1.0 mM NAD^+, and cell extract (0.5 to 1.0 mg). Assays were initiated by the addition of cell extract and incubated for 2 min at 37°C under a N_2 atmosphere. Enzyme activity was stopped by the addition of 1.0 ml of 0.5 N HCl. Bile acids were extracted and quantitated as previously described (7).

Immunoinhibition of bile acid 7-dehydroxylase activity was measured directly by incubating varying concentrations of purified IgG with cell extracts in 25 mM sodium acetate-MOPS (pH 7.5) for 30 min at 0–4°C under a N_2 atmosphere. After incubation, reaction mixtures were placed at 37°C and the reaction was immediately initiated by the addition of NAD^+ and [24-^{14}C]cholic acid. The assays were terminated and quantitated as described above. Controls included incubation with purified nonimmune IgG and no additions.

Purification of bile acid-inducible polypeptide

Cell extracts (~400 mg) were prepared as described above, except glycerol was omitted from the dialysis buffer. Soluble cell extract (20 mg/ml) was made 35% of saturation (0°C) with solid $(NH_4)_2SO_4$ and stirred for 60 min at 0°C. The precipitated material was removed by centrifugation (6000 *g* for 15 min at 0°C) and discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant fluid to 55% saturation (0°C) and stirred for 1 hr. The precipitate was obtained by centrifugation, dissolved in a minimal volume (2 to 3 ml) of 50 mM sodium phosphate (pH 6.8), and dialyzed in 1 liter of the same buffer for 12 to 14 hr at 4°C.

High performance liquid chromatography (HPLC) of the dialyzed $(NH_4)_2SO_4$ fraction was carried out using a Spherogel-TSK 3000 SW gel filtration column (60 × 0.75 cm) fitted with a precolumn (10 × 0.75 cm) (Toya Soda Manufacturing Co., Japan). The fractionation range of this column is between 300,000 and 12,000 daltons. The column was equilibrated and eluted with 50 mM sodium phosphate (pH 6.8) containing 100 mM NaCl. Approximately 25 mg of protein in a 1.0-ml injection volume was chromatographed per run. The flow rate was maintained at 0.85 ml/min and 1-min fractions were collected. Bile acid-inducible polypeptides were located and identified using SDS-PAGE as described below.

Fractions containing the bile acid-inducible polypeptides were pooled and dialyzed in 1 liter of 10 mM sodium phosphate (pH 6.8) for 12 to 14 hr at 4°C. The dialyzed material was then chromatographed on an Altex DEAE 545 HPLC column (15 × 0.75 cm). The column was initially equilibrated in 20 mM sodium phosphate (pH 6.0). Protein (20 mg/injection) was eluted by a gradient of NaCl from 0 to 125 mM in 5 min then increased to 500 mM in an additional 75 min. Flow rate was maintained at 0.85 ml/min and fractions were collected at 1-min intervals. Fractions containing the bile acid-inducible polypeptides (molecular weights 23,500, 18–19; 27,000, 33–34; 45,000, 26–27) were concentrated by ultrafiltration using an Amicon stirred cell with a YM-10 membrane and again chromatographed on a Spherogel-TSK 3000 SW column as described above. Fractions containing each polypeptide were dialyzed in 10 mM sodium phosphate (pH 6.8) and again chromatographed on a DEAE-545 column equilibrated with 20 mM sodium phosphate (pH 5.5). Protein was eluted using the same gradient as described above.

SDS-polyacrylamide gel electrophoresis

Purification of the cholic acid-inducible polypeptides was monitored by SDS-PAGE using a 10 to 20% exponential polyacrylamide gradient (4). Proteins were visualized by staining with Coomassie blue as described previously (4).

Preparation and purification of antibody to bile acid-inducible polypeptides

Antibodies were produced against proteins in fractions 19 to 23 (enzymatically active fractions) of the initial Spherogel-TSK 3000 SW column step, the purified 45,000 mol wt polypeptide, and partially purified 27,000 mol wt polypeptide. White New Zealand male rabbits were injected in the hind leg with 100 μg of purified 45,000 mol wt polypeptide, 100 μg of partially purified 27,000 mol wt polypeptide, or 1.5 mg of protein from fraction 19 to 23 emulsified in Freunds Complete Adjuvant. Maintenance injections were given monthly in Freunds Incomplete Adjuvant. Specific antibodies were initially detected by Ouchterlony diffusion (8).

Prior to initial injection of antigen, blood (30 to 50 ml) was withdrawn by cardiac puncture for the preparation of nonimmune serum. Blood was allowed to clot for 2 hr at room temperature and then overnight at

4°C. Blood was centrifuged for 10 min at 6000 *g* (4°C) and the serum was removed and stored in 5-ml aliquots at −40°C. Blood was withdrawn and sera were prepared in the same manner when needed for immune sera. IgG was purified from serum by protein A Sepharose affinity chromatography (9).

IgG produced against the proteins in the Spherogel-TSK 3000 SW gel filtration fractions 19 to 23 (TSK IgG) was further purified to remove IgG made in response to noninducible proteins. This was accomplished by immunoadsorption of TSK IgG with uninduced extract coupled to Sepharose 4B by cyanogen bromide coupling. Uninduced extract to be coupled to Sepharose 4B was prepared as described above except that the protein was dialyzed in 10 mM sodium phosphate (pH 7.4) containing 0.15 M NaCl. Sepharose 4B (12 ml packed volume) was washed three times in distilled water and suspended in 10 ml of distilled water. Cyanogen bromide (1.85 g) was dissolved in 12 ml of distilled water and added to the Sepharose 4B solution. The solution was gently stirred and the pH was maintained at 10.5 to 11.0 with 6 N NaOH. The activation reaction was completed when the pH stabilized between 10.5 and 11.0. The CNBr-activated Sepharose 4B was rapidly washed with 300 to 500 ml of 10 mM sodium phosphate (pH 7.4) containing 0.15 M NaCl. The packed CNBr-activated Sepharose 4B was transferred to a screw-capped tube containing 105 mg (6.4 ml) of protein. This suspension was shaken overnight at 4°C.

The protein-conjugated Sepharose 4B was centrifuged, washed two times in 0.1 M $NaHCO_3$ (pH 8.5), and suspended in 12 ml of 0.2 M glycine (pH 8.0). After 2 hr at room temperature, the coupled Sepharose 4B was washed in 0.1 M sodium acetate (pH 4.0) containing 1.0 M NaCl. This material was then washed in 0.01 M Tris-HCl (pH 8.0) containing 1.0 M NaCl and stored in 0.01 M sodium phosphate (pH 7.4) containing 0.15 M NaCl and 0.02% NaN_3 at 4°C.

TSK IgG was chromatographed on a 2 × 1 cm column of uninduced extract conjugated Sepharose 4B equilibrated with 100 mM sodium phosphate containing 500 mM NaCl. The eluent was collected and pooled and is referred to as TSK-absorbed IgG.

Immunoprecipitation

[^{35}S]Methionine-labeled polypeptides from bile-acid induced and uninduced cultures of *Eubacterium* sp. V.P.I. 12708 were prepared as described previously (4). Immunoprecipitation of [^{35}S]-labeled proteins from bile acid-induced and uninduced cell extracts was carried out according to the procedure of Jones (10). Commercially prepared *Staphylococcus aureus* Cowan I (Pansorbin) was washed in 10 mM sodium phosphate (7.2) containing 0.15 mM NaCl; 2 mM L-methionine; 0.5% NP-40, and 0.02% sodium azide (SAC buffer). After the second wash, *S. aureus* was suspended to its original volume in SAC buffer containing 1 mg/ml ovalbumin. [^{35}S]Methionine-labeled proteins (100,000 cpm) were incubated with 100 μl of IgG (approximately 0.3 mg) for 30 min at 4°C. *S. aureus* (50 μl of a 10% solution) was added to the IgG cell extract mixture and incubated for an additional 15 min. The immunoprecipitation reaction was terminated by the addition of 3 ml of SAC buffer. The *S. aureus* cells were centrifuged in an IEC clinical centrifuge (5 min). The cell pellet was suspended and washed two additional times in 3 ml of SAC buffer. Cells were then pelleted in an Eppendorf microcentrifuge, suspended in 50 μl of SDS sample buffer, and boiled for 5 min. *S. aureus* cells were removed by centrifugation and then the solubilized immunoprecipitated [^{35}S]methionine-labeled polypeptides were analyzed by SDS-PAGE as described above. Gels were impregnated with Enhance (New England Nuclear) and [^{35}S]methionine-labeled polypeptides were visualized using Kodak X-omat RP-1 film exposed at −70°C.

Chemicals

[24-^{14}C]Cholic acid (50 mCi/mmol), [^{35}S]L-methionine (>800 Ci/mmol), and Enhance were purchased from New England Nuclear Corp. Nonidit P-40 (NP-40) was obtained from Sigma Chemical Co. Electrophoresis reagents and SDS-PAGE molecular weight markers were purchased from Bio-Rad Laboratories. Pansorbin was obtained from Calbiochem. NAD^+ was purchased from P-L Biochemicals Inc. All other chemicals were of the highest grade commercially available.

RESULTS

Preparation of antibodies to bile acid-inducible polypeptides

We previously demonstrated (4) that bile acid 7α-, 7β-dehydroxylase and Δ^6 reductase activities all co-elute from a Spherogel-TSK SW 3000 HPLC gel filtration column. Therefore, fractions (number 19 to 23) containing these activities were pooled and injected into a rabbit as described in Materials and Methods.

Ouchterlony double diffusion plates were initially used to determine whether antibody had been produced against the bile acid-inducible polypeptides in soluble cell extracts of *Eubacterium* sp. V.P.I. 12708. IgG prepared against fractions 19 to 23 (TSK antibody) was initially adsorbed with uninduced cell extract coupled to Sepharose 4B. At least two precipitin bands were observed between TSK antibody and induced soluble extracts (data not shown). No detectable precipitin bands were observed with uninduced cell extracts.

In order to further examine antibody specificity and to determine whether other polypeptides were co-precipitated, [^{35}S]-labeled proteins were immunoprecipitated. TSK and TSK-adsorbed IgG preparations were individually incubated with [^{35}S]-labeled polypeptides from induced and uninduced cultures. Immune complexes were precipitated with *S. aureus* (Cowan I) and examined by SDS-PAGE (**Fig. 1**). The TSK-adsorbed IgG immunoprecipitated the 45,000, 27,000, and 23,500 molecular weight polypeptides from induced cell extracts (Fig. 1, lane E). No detectable polypeptides were precipitated from [^{35}S]-labeled uninduced extract (Fig. 1, lane F). No detectable polypeptides were precipitated with nonimmune IgG.

Immunoinhibition studies

TSK IgG contains antibody to 45,000, 27,000, and perhaps a small amount to the 23,500 mol wt polypeptides. Because this IgG was prepared against HPLC column fractions containing bile acid 7-dehydroxylase activity, this preparation should have contained antibodies to this enzyme. To determine whether these antibodies inhibited bile acid 7α-dehydroxylase activity, immunoinhibition studies were carried out. Varying amounts of IgG from different preparations were incubated with cell extracts containing bile acid 7-dehydroxylase activity. Enzyme activity was measured in the IgG-extract mixture

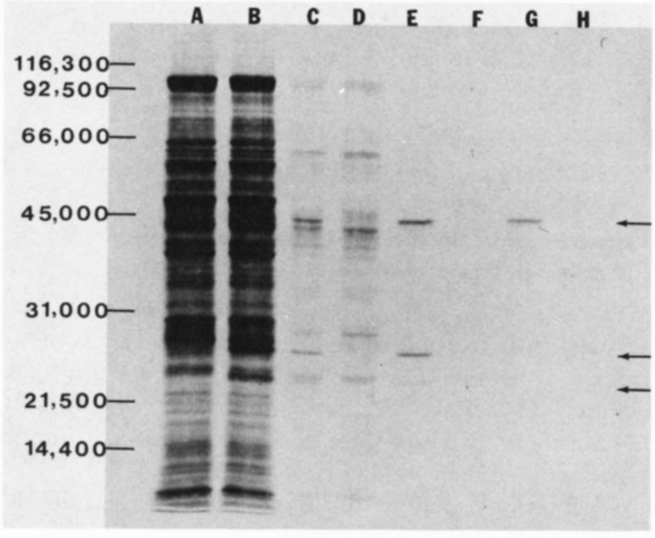

Fig. 1. Immunoprecipitation of soluble [^{35}S]methionine-labeled polypeptides from cholic acid-induced and uninduced cultures of *Eubacterium* sp. V.P.I. 12708. Arrows indicate positions of cholic acid-induced polypeptides. Lane A, uninduced cell extract; lane B, cholic acid-induced cell extract; lane C, cholic acid-induced cell extract and TSK IgG; lane D, uninduced cell extract and TSK IgG; lane E, cholic acid-induced cell extract and TSK-adsorbed IgG; lane F, uninduced cell extract and TSK-adsorbed IgG; lane G, cholic acid-induced cell extract and IgG prepared against the purified 45,000 mol wt polypeptide; lane H, uninduced cell extract and IgG prepared against the purified 45,000 mol wt polypeptide.

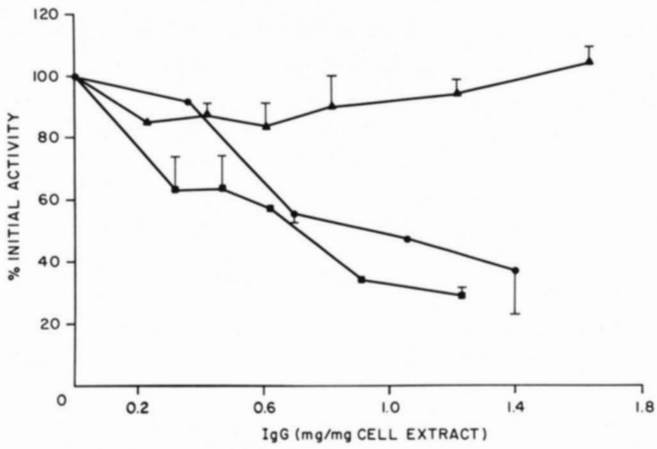

Fig. 2. The effect of TSK, TSK-adsorbed, and non-immune IgG on bile acid 7α-dehydroxylase activity in cell extracts of *Eubacterium* sp. V.P.I. 12708. Various amounts of TSK-IgG (●), TSK-adsorbed (■), or non-immune IgG (▲) were preincubated (30 min) with cholic acid-induced cell extract of *Eubacterium* sp. V.P.I. 12708 at 4°C under a N_2 atmosphere. Bile acid 7-dehydroxylase activity was then assayed as described in Materials and Methods.

following a 30-min preincubation. The results showed that both the TSK and TSK-adsorbed IgG inhibited bile acid 7α-dehydroxylase activity in a concentration-dependent manner (**Fig. 2**), suggesting that one or more of these three polypeptides are involved in 7α-dehydroxylation.

Purification and preparation of antibody to the 45,000 mol wt protein

Because the 45,000 mol wt polypeptide was immunoprecipitated by TSK-absorbed antibody, it was purified for further study. Purification of the 45,000 molecular weight polypeptide was accomplished using $(NH_4)_2SO_4$ fractionation, HPLC gel filtration, and HPLC-DEAE chromatography as described in Materials and Methods. Identification of the 45,000 mol wt polypeptide and evaluation of purity was monitored by SDS-PAGE for each step of the purification (**Fig. 3**). The $(NH_4)_2SO_4$ precipitate (35 to 55%) was applied to a HPLC gel filtration column and fractions 19 to 26 (containing bile acid-inducible polypeptides) were pooled, dialyzed, and applied to a HPLC-DEAE column (pH 6.0). DEAE-HPLC chromatography resolved the 45,000, 27,000, and 23,500 molecular weight polypeptides that eluted in fractions 26 and 27 (0.23 M NaCl), 33 and 34 (0.26 M NaCl), and 18 and 19 (0.19 M NaCl), respectively (**Fig. 4**). The next step in the purification of the 45,000 molecular weight polypeptide involved a second HPLC gel filtration step followed by a second HPLC-DEAE (pH 5.5). The final HPLC-DEAE step showed a protein peak eluting at 25 to 26 min (0.23 M NaCl) corresponding to the 45,000 mol wt polypeptide. Further analysis of the DEAE column fractions by SDS-PAGE and iso-

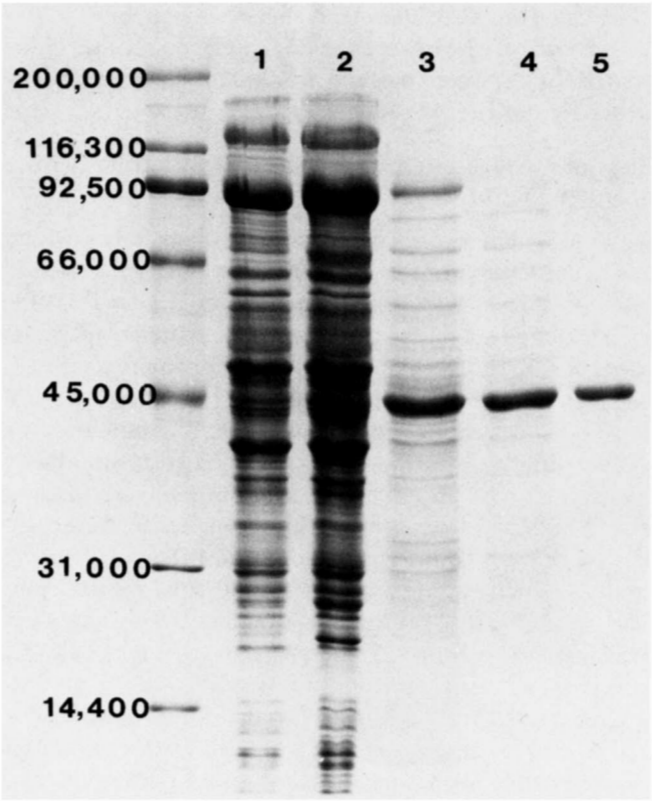

Fig. 3. Purification of the 45,000-dalton bile acid-inducible polypeptide from *Eubacterium* sp. V.P.I. 12708. Lane 1, uninduced soluble cell extract (75 μg); lane 2, induced soluble cell extract (75 μg); lane 3, pooled fractions 26 and 27 from HPLC-DEAE column (pH 6.0); lane 4, pooled fractions 21, 22, and 23 from second TSK 3000 SW gel filtration column; lane 5, pooled fractions 25 and 26 from HPLC-DEAE column (pH 5.5).

electric focusing revealed a second 45,000 mol wt protein eluting just prior to the 45,000 mol wt polypeptide which had the same molecular weight but a pI of 4.03 compared to 4.1.

The purified 45,000 mol wt polypeptide was injected into a rabbit to raise specific antibody to this protein. A single precipitin band was observed between antibody (IgG) prepared against the 45,000 mol wt polypeptide and the purified 45,000 mol wt protein (**Fig. 5**, wells 3 and 6). Moreover, soluble cell extracts prepared from bile acid-induced cultures of *Eubacterium* sp. V.P.I. 12708 also showed a single precipitin band (Fig. 5, wells 1 and 4). There was a line of identity between the purified protein and induced cell extract. There was no detectable precipitin band observed with uninduced extract (Fig. 5, wells 2 and 5) or if nonimmune IgG was used in center well (data not shown). IgG raised against the 45,000 mol wt polypeptide precipitated a single polypeptide of 45,000 molecular weight from [^{35}S]-labeled induced extracts (Fig. 1, lane G) but not uninduced extracts (Fig. 1, lane H). No detectable polypeptides were precipitated with nonimmune IgG. When the immunoprecipitated 45,000 mol wt polypeptide was subjected to isoelectric focusing, a single polypeptide was observed with a PI of 4.1. This was the same pI as that of the purified polypeptide.

Immunoinhibition studies showed no detectable inhibition of bile acid 7α-dehydroxylase activity. Bile acid products from enzymatic reaction mixtures containing IgG prepared against the 45,000 mol wt polypeptide were chromatographed on $AgNO_3$-treated thin-layer

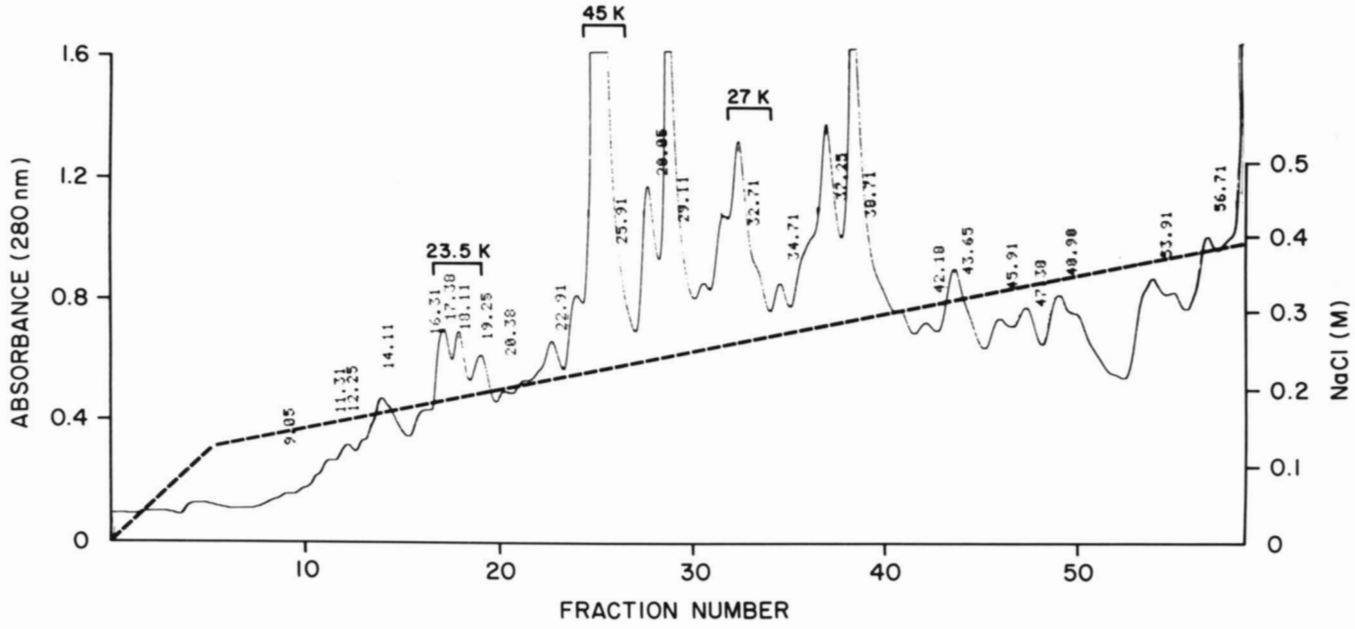

Fig. 4. HPLC-DEAE elution profile of partially purified cell extracts from *Eubacterium* sp. V.P.I. 12708. The solid line represents A_{280} and the dashed line represents the concentration of NaCl. Brackets indicate the positions of bile acid-inducible polypeptides. The vertical numbers represent retention times of protein peaks. Conditions of protein elution are described in Materials and Methods.

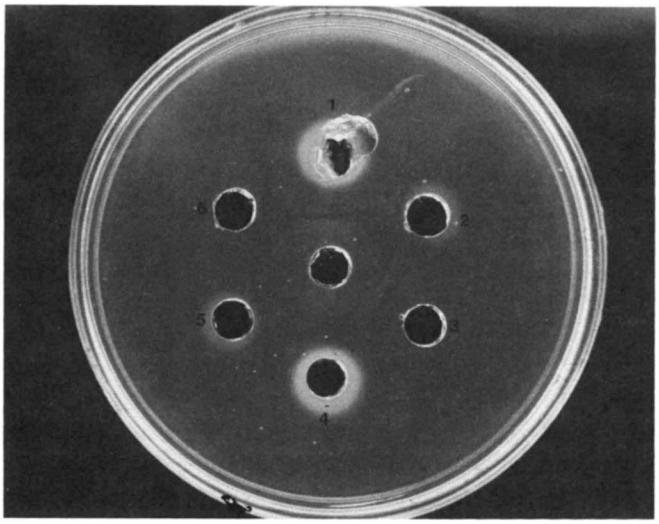

Fig. 5. Ouchterlony double diffusion plate containing IgG (0.64 mg) prepared against the purified 45,000 mol wt bile acid-inducible polypeptide in the center well. The outer wells contained: cholic acid-induced soluble cell extract (4.7 mg), wells 1 and 4; uninduced soluble cell extract (1.9 mg), wells 2 and 5; purified 45,000 mol wt polypeptide (0.02 mg), wells 3 and 6.

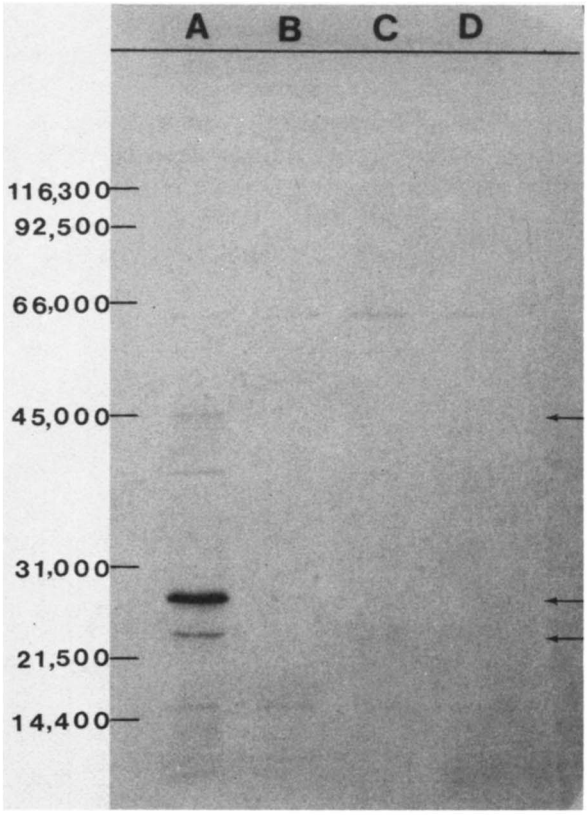

Fig. 6. Immunoprecipitation of soluble [^{35}S]methionine-labeled polypeptides from cholic acid-induced and uninduced extracts prepared from *Eubacterium* sp. V.P.I. 12708. Arrows indicate positions of cholic acid-induced polypeptides. Lane A, cholic acid-induced cell extract and adsorbed IgG against the 27,000 mol wt polypeptide; lane B, cholic acid-induced cell extract and nonimmune IgG; lane C, uninduced cell extract and adsorbed IgG against the 27,000 mol wt polypeptide; lane D, uninduced cell extract and nonimmune IgG.

chromatograms to allow for the detection of the Δ^6-intermediate. However, no Δ^6-intermediate was observed, suggesting that the 45,000 mol wt protein is probably not the Δ^6-reductase.

Partial purification and preparation of antibody to the 27,000 mol wt protein

The 27,000 mol wt polypeptide was partially purified using the same procedure described to purify the 45,000 mol wt polypeptide, except that the final DEAE-HPLC step was omitted due to poor recovery of the polypeptide. The 27,000 mol wt polypeptide was approximately 80% pure as estimated by SDS-PAGE and was used to raise antibody in rabbits. IgG against the 27,000 mol wt polypeptide was purified and adsorbed with uninduced extract. This antibody preparation immunoprecipitated the 27,000 mol wt polypeptide from radiolabeled induced but not uninduced cell extracts (**Fig. 6**, lanes A and C). Small amounts of the 45,000 (8% of total cpm) and 23,500 (13% of total cpm) mol wt peptides coprecipitated with the 27,000 (79% of total cpm) mol wt polypeptide. Immunochemical staining (Western Blot analysis) of SDS-PAGE of induced cell extracts showed only a single immunoreactive band at 27,000, indicating no detectable antibodies to the 23,500 or 45,000 mol wt proteins (data not shown).

Immunoinhibition studies showed a concentration-dependent inhibition of 7α-dehydroxylase activity (**Fig. 7**) with antibody to the 27,000 mol wt polypeptide.

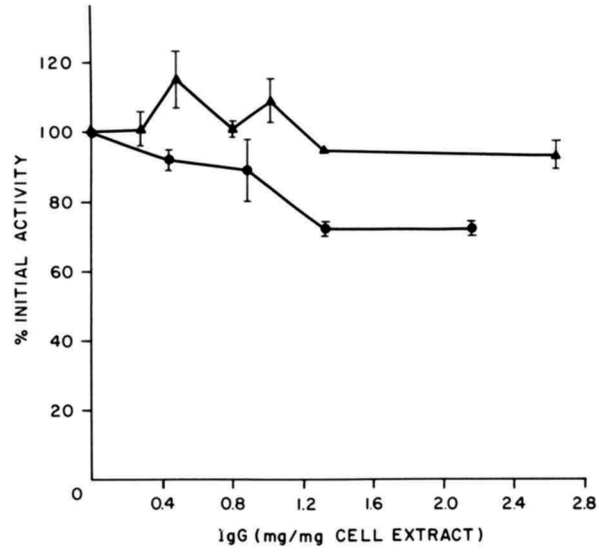

Fig. 7. The effect of IgG against the 27,000 mol wt polypeptide on bile acid 7α-dehydroxylase activity in cell extracts of *Eubacterium* sp. V.P.I. 12708. Varying amounts of non-immune IgG (▲) and IgG against the 27,000 mol wt polypeptide (●) preadsorbed with uninduced cell extract was preincubated (30 min) with cholic acid-induced cell extract of *Eubacterium* sp. V.P.I. 12708 at 4°C under a N_2 atmosphere. Bile acid 7-dehydroxylase activity was then assayed as described in Materials and Methods.

DISCUSSION

Bile acid 7-dehydroxylase from *Eubacterium* sp. V.P.I. 12708 is very labile and recalcitrant to purification by conventional purification techniques that rely on measuring enzyme activity. Therefore we have approached this problem by purifying cholic acid-inducible polypeptides that co-eluted with bile acid 7-dehydroxylase activity through an anaerobic HPLC gel filtration column. White et al. (4) previously reported the presence of bile acid-inducible polypeptides in whole cells at 77,000, two at 56,000, 27,000 and 23,500 mol wt. In the present study we used column fractions containing enzyme activity to raise antibodies to bile acid-inducible polypeptides. When IgG was prepared from rabbits injected with these proteins and adsorbed with uninduced cell extracts, three polypeptides with molecular weights of approximately 45,000, 27,000 and 23,500 were immunoprecipitated (Fig. 4, lanes E and F). The 23,500 mol wt polypeptide appeared in very low concentration relative to the 27,000 and 45,000 mol wt proteins. Unexpectedly, we did not detect a bile acid-inducible 77,000 or 56,000 mol wt polypeptide. Additional experiments showed that this antiserum inhibited bile acid 7α-dehydroxylase activity in a concentration-dependent manner (Fig. 2). These data indicate that one or more of these three polypeptides are involved in 7α-dehydroxylation.

In order to define the role of each inducible polypeptide in bile acid 7-dehydroxylation, we purified the 45,000 mol wt protein and partially purified (80%) the 27,000 mol wt protein. When antibody was raised to the 45,000 mol wt polypeptide and used in immunoprecipitation experiments, a single bile acid-inducible polypeptide was observed (Fig. 1, lanes G and H). These results suggest that this polypeptide is not tightly associated with other bile acid-inducible proteins. Immunoinhibition studies showed no detectable effect on cholic acid 7α-dehydroxylation activity. Therefore, the exact function of this protein in bile acid 7α-dehydroxylation is not yet clear. However, it does not eliminate some role for this protein in this biotransformation reaction.

Antibody, specific for the 27,000 mol wt polypeptide, immunoprecipitated that protein as well as small amounts of the 45,000 and 23,500 mol wt polypeptides (Fig. 6). Immunochemical staining showed that this antibody is highly specific for the 27,000 mol wt protein. These results suggest that small amounts of these proteins may be complexed with the 27,000 mol wt polypeptide in cell extracts. Moreover, IgG prepared against the 27,000 mol wt polypeptide inhibited 7α-dehydroxylase activity in a concentration-dependent manner (Fig. 7) which suggests that the 27,000 mol wt polypeptide is involved in enzyme catalysis.

The preparation of specific antibodies to bile acid 7-dehydroxylase from *Eubacterium* sp. V.P.I. 12708 is an important step in obtaining the necessary reagents for use in determining by immunological techniques the cellular location and distribution of this enzyme among other bacteria, as well as screening a genomic clone bank for genes coding for this enzyme. Additional characterization of the 45,000, 27,000 and 23,500 mol wt polypeptides will be required to elucidate the role each plays in the 7-dehydroxylation of bile acids.

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