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PURIFICATION AND CHARACTERIZATION OF A CYTOCHROME P-450 ISOZYME ISOLATED FROM THE LIVER OF RATS PRETREATED WITH CLOFIBRATE

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1- Using a methodology of purification consisting of only one chromatographic step (phenyl-Sepharose) we have purified a cytochrome P-450 isozyme from liver of clofibrate - treated rats. It was called cytochrome P-450 clo. 2- A single polypeptide of mol.wt. 50,000 was visible after sodium dodecyl sulphate polyacrylamide gel electrophoresis. 3- Antiserum raised against the pure enzyme specifically recognized P-450 clo and inhibited more than 90% of the 11- and 12- laurate hydroxylase activities present in clofibrate - treated rats. 4- Clofibrate treatment of the rats resulted in a six fold increase in microsomal cytochrome P-450 clo as judged by immunochemical quantification. This result is in agreement with the increase of laurate hydroxylase activity after treatment by clofibrate.

Several compounds including hypolipidaemic drug clofibrate have been shown in rodents to produce hepatic peroxisome proliferation (1), increases in the enzymes of peroxisomal fatty acid β- oxidation and microsomal cytochrome P-450 dependent hydroxylations (2,3). Lauric acid is hydroxylated at the 11- and 12- positions by microsomes from liver. Preferential induction of 11- hydroxylation by phenobarbital treatment and preferential inhibition by CO, SKF 525 A and metyrapone suggest that different cytochrome P-450 isozymes catalyse the 11- and 12- hydroxylations in the liver (4). Nevertheless, a cytochrome P-450 dependent laurate 12- hydroxylase has been recently isolated from rats given clofibrate by two different groups (5,6). The identity of both these isoenzymes is not yet established. The present paper reports the purification and characterization of a form of cytochrome P-450 from clofibrate - treated rat liver microsomes.

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MATERIALS AND METHODS

Sprague Dawley male rats weighing 180-200g were given clofibrate by an intraperitoneal injection at a dose of 400mg/Kg/day for 4 days. Liver microsomes used for catalytic and immunochemical studies were prepared as previously described (7).

Purification. The first step of the enzyme purification (microsomal preparation, membrane solubilization and ammonium sulphate precipitation) corresponded to the procedure of Knowles and Burchell (8). The ammonium sulphate precipitate was dissolved in a minimal volume of buffer (100mM potassium phosphate, pH 7.4 containing 1mM EDTA, 1mM DTT, 20% glycerol, and 0.05% Lubrol PX) and subjected to phenyl-Sepharose CL-4B chromatography. A linear gradient of 0.05 to 1% of Lubrol was applied to the column. Ten milliliter fractions were collected and an aliquot was checked on SDS-PAGE for homogeneity and assessed by 417nm absorption for cytochrome P-450 content. As a result of this procedure, a fraction of cytochrome P-450 was eluted by 1% Lubrol.

General assays and reconstitution experiments. Cytochrome P-450 content was determined as described by Omura and Sato (9). Protein determination was performed according to Lowry et al. (10) and to Dulley and Grieves for detergent containing fractions (11). Lauric acid hydroxylation and analysis of 11- and 12- hydroxylated products were performed as described by Fan et al. (12) and modified as in (13). Fractions were assayed for laurate 11- and 12-hydroxylase activities after removal of 90% of the non ionic detergent with Bio-Beads SM-2 column (1g beads per mg protein) (14,15). The reconstituted lauric acid hydroxylation assay system contained 0.2 nmol of cytochrome P-450, 0.2 nmol of NADPH - cytochrome P-450 reductase, 0.1mg of dilauroyl phosphatidyl choline, 0.05 mg of sodium cholate and was preincubated for 10 min in presence of substrate before starting the assay (16).

Preparation of polyclonal antibodies. Female New Zealand white rabbits were inoculated subcutaneously with 50µg of antigen mixed 1:1 (v/v) with complete Freund's adjuvant. Two similar booster injections were given every week for 2 weeks. Antisera were collected during the 4th week. IgG were purified using a protein A-Sepharose CL-4B column according to the procedure described by Weatherill et al. (17). The other rat cytochromes P-450 i.e. UT-A, PB-B, BNF-B (the major hepatic cytochromes P-450 from untreated rats, phenobarbital and benzo-β-naphthoflavone - treated rats respectively) and corresponding antisera were kindly provided by Ph. Beaune and J. P. Flinois, and were prepared as previously described (18).

Electrophoresis, immunoblot and Ouchterlony double - diffusion analysis. 10 - 20µg of microsomal proteins or 0.5 - 5 pmol of purified cytochrome P-450 were separated in the discontinuous Laemmli system (19) with a 9% concentration of acrylamide instead of 7.5%. Proteins were either revealed by silver nitrate (20) or transferred to nitrocellulose sheets according to previously described procedures (21-24). Immunoblots were stained with 4-chloro-1-naphthol. (23-24) Ouchterlony double - diffusion analysis (25) were carried out in culture dishes containing 1% agarose in 0.9% Nacl.

Immunoinhibition. Microsomes from control or clofibrate - treated rats were incubated for 20 min at 25° with various amounts of IgG from sera of immunized or non immunized rabbits before starting the reactions. Activities are expressed as percentages of the activity determined without IgG fractions.

RESULTS

Purification of P-450 clo. After hydrophobic affinity chromatography of ammonium sulphate precipitate on a phenyl-Sepharose CL-4B column, a cytochrome P-450 - containing fraction was isolated in fractions eluted with a buffer

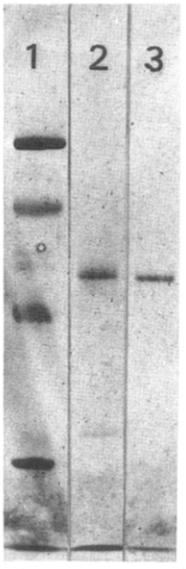


Fig 1. SDS polyacrylamide gel electrophoresis. Migration proceeds from top to bottom. lane 1, LMW calibration Kit proteins from Pharmacia including phosphorylase b (Mr = 94,000), Albumin (Mr = 67,000), ovalbumin (Mr = 43,000), trypsine inhibitor (Mr = 20,000); lane 2 , P-450 clo (5μg) lane 3 , P-450 UT-A (5μg).

containing 1% Lubrol PX. This fraction contained a major polypeptide of monomeric Mr 50kD as judged by Na Dod SO4-polyacrylamide gel electrophoresis (Fig. 1). Fractions containing the protein band of apparent Mr 50 kD were designated as P-450 clo. The overall yield of P-450 clo on the basis of the spectrally determined P-450 present in clofibrate-treated rat liver microsomes was 7.5%. A specific content of 3.0 nmol P-450. mg^{-1} protein was determined by a CO-difference spectrum and Lowry protein determination. Several possibilities may explain this low specific activity, namely loss of prosthetic heme during purification, insufficient reduction of oxidized heme iron (26), over estimation of the protein content by the Lowry procedure (27) or contamination by proteins with the same molecular weight. On the basis of subsequent immunochemical estimations of the level of P-450 clo in clofibrate rat liver microsomes (Table I), P-450 clo was purified about 2.5 fold during this procedure. The absorption maximum in reduced carbon monoxide difference spectra was at 450nm.

TABLE I- CYTOCHROME P-450 clo CONTENT IN CONTROL AND CLOFIBRATE - TREATED RAT MICROSOMES

	Control	Clofibrate treated	fold increase
cytochrome P-450 [a] (nmol.mg^{-1} Protein) n = 6	0.87 ± 0.08	1.60 ± 0.13	1.85
11-hydroxylation [b] (nmol.min^{-1}.mg^{-1} Protein) n = 6	0.43 ± 0.10	1.38 ± 0.29	3.20
12-hydroxylation [b] (nmol.min^{-1}.mg^{-1} Protein) n = 6	0.56 ± 0.17	5.27 ± 1.30	9.40
Cytochrome P-450 clo [c] (nmol.mg^{-1} Protein) (triplicate determination)	0.12	0.75	6.40

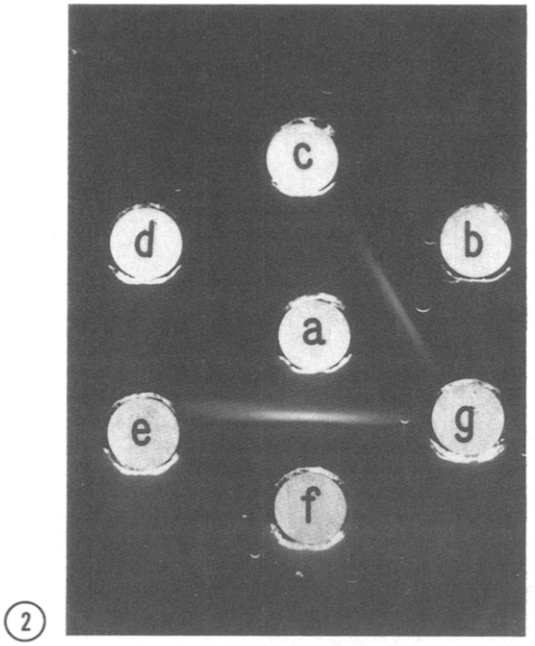
[a] spectral determination

[b] enzymatic determination of laurate hydroxylase activity

[c] immunochemical determination

Reconstitution of lauric acid hydroxylase activity. P-450 clo catalyzed the 11- and 12- hydroxylations of lauric acid in a reconstituted system, respectively 0.11nmol and 0.25nmol .min^{-1} per nmol P-450. It is generally acknowledged that microsomal cytochrome P-450 dependent hydroxylations can not be directly compared to in vitro reconstituted activities. Residual detergents, solvent effects and/ or complexities of the interactions between the different components may be involved (28). Because of the low level of activity in the reconstituted system, inhibition studies using the antibody anti-P-450 clo have not been performed.

Electrophoretic and immunochemical characterization. By comparison to known molecular weight standards, the molecular weight of P-450 clo is estimated to be 50kD and is similar in electrophoretic mobility to P-450 UT-A (Fig 1). As judged by Ouchterlony double-diffusion the anti P-450 clo gave a single immunoprecipitation line on reaction with its homologous antigen. In addition, there was no cross-reaction with P-450 UT-A or P-450 PB-B (Fig.2). With the



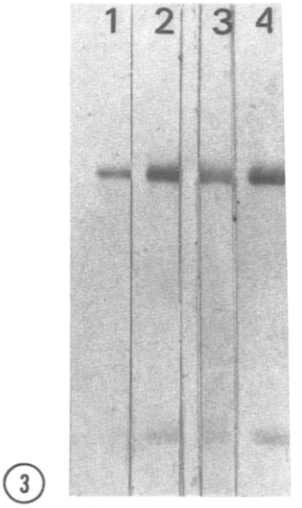


Fig 2. Double-immunodiffusion analysis of P-450 clo. a, anti P-450 clo serum (2.5μl); b and f, P-450 clo (40 pmoles); c, P-450 PB-B (40 pmoles); d and g, P-450 UT-A (40 pmoles); e, Epoxide hydrolase (5μg).

Fig 3. Immunnoblot of rat liver microsomal proteins and cytochrome P-450 clo immunoreactive with anti P-450 clo. Lane 1 and 2, 0.75 and 3.75 pmoles of cytochrome P-450 clo; lane 3 and 4, 9 pmoles of rat liver microsomal cytochrome P-450 were loaded (lane 3 untreated rats, lane 4 clofibrate treated rats). the nitrocellulose sheet was incubated with a 1/100 dilution of rabbit antiserum raised to P-450 clo. The bands were densitometered.

Western blotting technique, anti P-450 clo was found to recognize only the homologous antigen but not other cytochrome P-450 isozymes including P-450 UT-A, P-450 PB-B, and P-450 BNF-B confirming the results of Ouchterlony technique. In liver microsomes from untreated or clofibrate - treated rats, anti P-45 clo recognized a single protein band of the same apparent molecular weight as that of purified P-450 clo (Fig. 3). By comparison with a standard curve, the content of P-450 clo was 0.12nmol P-450 per mg protein in untreated rat microsomes and 0.75nmol per mg protein in clofibrate - treated rat microsomes (Table I). The increase observed in P-450 clo content after clofibrate treatment, is in agreement with the raise of total (11 + 12) lauric acid hydroxylase activity (Table I). P-450 clo constitutes respectively about 14% and 47% of the spectrally determined cytochrome P-450 in control and clofibrate - treated rat microsomes (Table I).

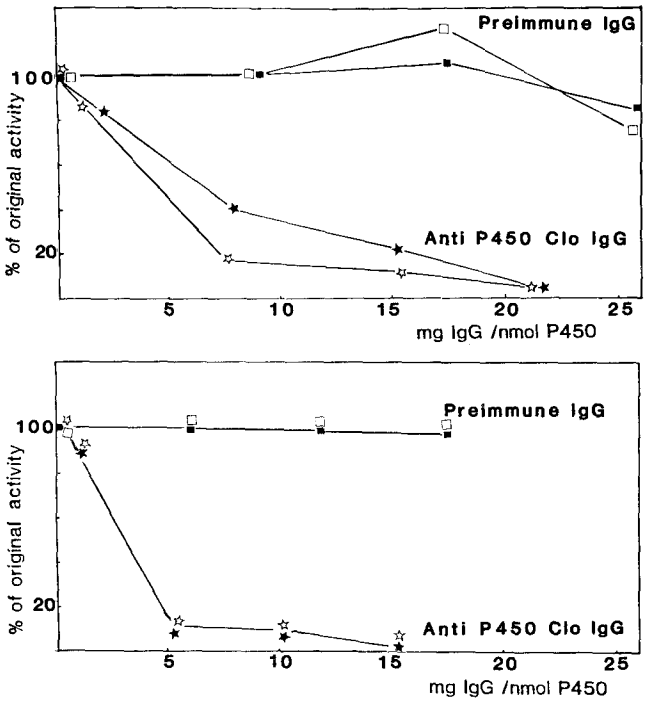


Fig 4. Inhibition of laurate hydroxylation by an antibody raised to cytochrome P-450 clo. Microsomes isolated from control (A) or clofibrate treated (B) rats were incubated with various amounts of anti cytochrome P-450 clo IgG and the enzymatic activity was determined as described in Materials and Methods.
☆---□ 11- hydroxylation and ★---■ 12- hydroxylation

Immunoinhibition of laurate hydroxylase activity. To determine the contribution of P-450 clo to the microsomal laurate hydroxylase activity, an IgG fraction prepared from rabbit antisera raised against P-450 clo was examined for its ability to inhibit the laurate hydroxylase activity catalysed by microsomes prepared from clofibrate -treated rats and untreated rats. Anti P-450 clo inhibited the 11- and 12- laurate hydroxylations in a concentration-dependent manner (Fig.4). In microsomes from clofibrate - treated rats, anti P-450 clo was able to inhibit the 11- and 12- laurate hydroxylations by 90% at a ratio of 5mg IgG per nmol P-450. A residual activity of 35% and 40% for the 11- and 12-hydroxylation respectively was observed at the same ratio in untreated rats microsomes. Rabbits preimmune IgG fractions have no effect on the enzyme activity catalysed by liver microsomes. Thus these immunoinhibition experiments indicated that P-450 clo is responsible for almost all of the

laurate hydroxylase activity in clofibrate - treated rats. However in control rats, the contribution of other cytochrome P-450 isozymes to hydroxylation of lauric acid is more important. Moreover the pattern of the inhibition is similar for 11- and 12- hydroxylations.

DISCUSSION

Using electrophoresis method as the basis for microsomal P-450 purification, we have obtained a form of hepatic cytochrome P-450, designated as P-450 clo, that is involved in the 11- and 12- hydroxylations of lauric acid. This cytochrome P-450 is present in non induced rat liver and its level is markedly elevated after clofibrate administration in agreement with the increase in lauric acid hydoxylase activity. (Table I). Two different groups have recently reported the purification of hepatic cytochrome P-450 isozymes involved in the 11- and 12- hydroxylations of lauric acid and induced by clofibrate treatment (5,6). Using the immunoquantification by Western blotting technique, we determined the amount of P-450 to be 14% of the total cytochrome P-450 population in control rats and 47% in clofibrate - treated rats. These results are similar to those reported by Bains et al. (29) Moreover in agreement with these authors, we have displayed only a single polypeptide band by Western blotting with microsomes from either control or clofibrate treated rats. This pattern is different from that reported by Hardwick et al. (6) who showed the presence of two clofibrate - induced proteins recognized by their antibodies in the liver of control and clofibrate treated rats. On the other hand, Bains et al. (29) and Hardwick et al. (6) reported a differential inhibition in the 11- and 12- hydroxylations of lauric acid. In contrast, our studies showed a similar inhibition for the 11- and 12-hydroxylations, suggesting that anti cytochrome P-450 clo recognizes either an unique cytochrome P-450 or antigenic sites common to two or more cytochromes P-450 involved in the 11- and 12-hydroxylations of lauric acid. In conclusion, on the basis of the immunochemical characteristics of cytochrome P-450 clo, we believe that it is probably different from the previously isolated forms (5,6). However the final evidence

of difference or similarity should be brought by the protein sequence determination.

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