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COMPARISON OF HIGHLY-PURIFIED MICROSOMAL CYTOCHROMES P-450 AND NADPH-CYTOCHROME P-450 REDUCTASES BY PEPTIDE MAPPING

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SUMMARY

Considerable differences were observed among several cytochrome P-450 species upon mapping of proteolytic digests, although certain preparations derived from rabbits were not distinguished by this technique. Several NADPH-cytochrome P-450 reductases gave peptide maps rather similar to each other. The results support and extend other evidence that microsomal cytochromes P-450 are not necessarily closely-related polypeptides.

INTRODUCTION

The roles of P-450[1] and NADPH-cytochrome P-450 reductase (EC 1.6.2.4.) in the microsomal biotransformation of xenobiotics have been established (1,2); much attention has been directed toward the subject of the multiplicity of these enzymes and its role in metabolism. Barbituates and polycyclic hydrocarbons were first shown to preferentially induce different enzyme activities and spectral properties of the P-450s (2,3). More recently, evidence for the presence of multiple P-450s in microsomal preparations has been obtained by electrophoretic (4), genetic (5), and immunological (6,7) techniques. P-450s have been purified to varying degrees and differences in the isolated enzymes have been demonstrated using substrate specificities (8-11), electrophoresis (8-12), spectra (8-12), immunological studies (7, 11, 13-16), and amino acid compositions (12,16). The multiplicity of NADPH-cytochrome P-450 reductase has been less thoroughly examined; however, Coon *et al* (15) have demonstrated the presence of electrophoretic variants of the rat liver enzyme.

Most of the above techniques, although valuable, do not address the

[1]Abbreviations: P-450, microsomal cytochrome P-450; PB, phenobarbital; 3MC, 3-methylcholanthrene; SDS, sodium dodecyl sulfate.

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question of how similar the primary structures of these enzymes are. The possibilities that the proteins may be highly similar and that various enzymes may be the result of proteolysis have not been completely ruled out, although the low extent of immunological cross-reactivity of certain P-450s (6,7,11,13-16) argues against such hypotheses. In this report, advantage was taken of the availability of highly-purified enzymes and a sensitive, convenient peptide mapping method to investigate similarities and differences between a number of purified P-450s and NADPH-cytochrome P-450 reductases.

METHODS

Enzyme Preparations

Liver microsomal P-450s were purified from PB- and 3MC-treated rats using previously described methods (8,16). The P-450s PB-B, PB-D, and 3MC-B (8) were homogeneous as judged by polyacrylamide gel electrophoresis in six different systems [(17,18,19), the acidic SDS and neutral cationic systems of Fairbanks and Avruch (20), and a modification of (17) using double strength buffer at the cathode]. Each P-450 showed a single Ouchterlony band against each of the immunoglobulin G fractions prepared from rabbits treated with PB-B and 3MC-B P-450s, yielded only a single N-terminal, and gave rise to linear double-reciprocal 7-ethoxycoumarin de-ethylase kinetic plots (16). Specific contents of the PB-B, PB-D, and 3MC-B preparations were 18, 14, and 17 nmol P-450 per mg protein, respectively. The PB-A preparation appeared homogeneous in the first five of the electrophoretic systems, but was judged to be a mixture of at least two proteins by electrophoresis in the sixth system, immunological reactivity with antibody prepared to 3MC-B P-450, and steady-state kinetics of 7-ethoxycoumarin de-ethylase activity (16).

NADPH-cytochrome P-450 reductases (11) and rabbit liver P-450s (8) were prepared as previously described. Bromelain-solubilized NADPH-cytochrome c reductase (21) was a gift of Dr. S. D. Aust, Michigan State University, East Lansing. All of these preparations appeared to be homogeneous as judged by SDS-polyacrylamide gel electrophoresis (8,11).

Peptide Mapping

Each protein (nominally 25 µg in 50 µl) was treated with *Staphylococcus aureus* V8 protease (Miles Laboratories; 0.5 µg), α-chymotrypsin (Miles Laboratories; 0.5 µg), or papain (Sigma; 0.05 µg) at 37° for 30 min using the procedure of Cleveland *et al* (22). (Optimum protease concentrations and incubation time were established in preliminary experiments using rabbit P-450 LM-2 and rat NADPH-cytochrome P-450 reductase.) One-half of each incubate was applied to a well of a 15% acrylamide slab gel (Hoefer, 0.75 mm x 10 cm) prepared according to Laemmli (17); electrophoresis (at 10°) was carried out at 10 mA/gel during stacking and 20 mA/gel during migration of the dye front in the separating gel. Staining was according to Fairbanks *et al* (23).

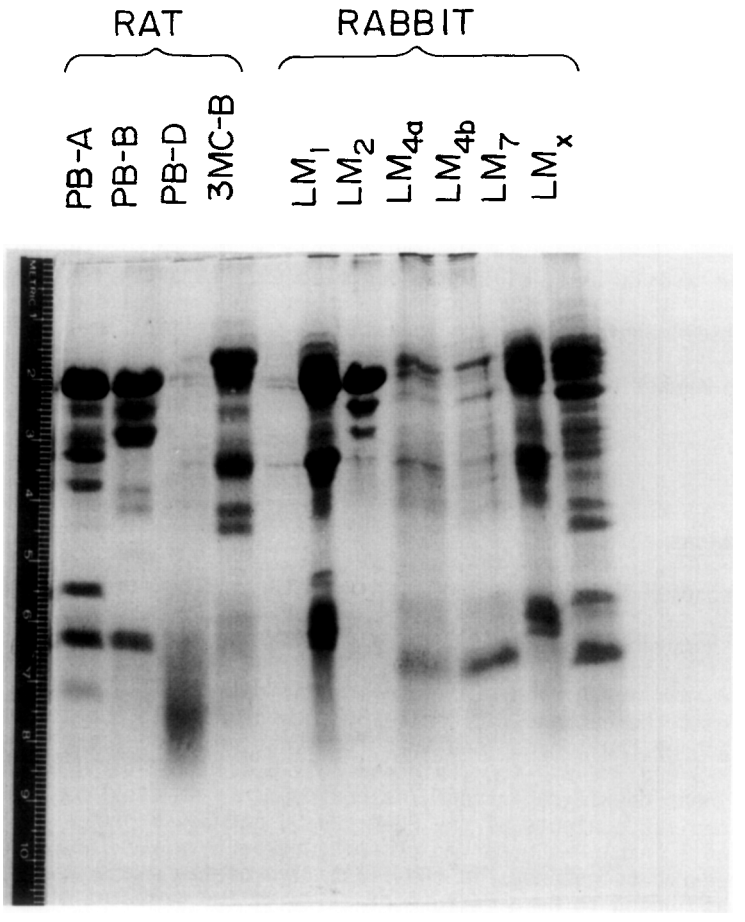

Figure 1. Peptide maps of P-450s after digestion with *S. aureus* V8 protease. The four tracks on the left are rat liver P-450s; the fifth track is the protease only; the six remaining tracks on the right are the rabbit P-450s. The anode was at the bottom of the gel.

RESULTS AND DISCUSSION

Rat liver P-450s PB-B, PB-D, and 3MC-B (for which the most evidence for homogeneity has been obtained) show extensive differences in their peptide maps (Figs. 1-3). In the chymotrypsin digests (Fig. 2), P-450s PB-B and 3MC-B (prepared by identical procedures from rats treated with different inducers) show some similarity with regard to major large peptides (2.5 and 3.5 cm); these results emphasize the need to use several different proteases in such work. Since the P-450 PB-A preparation is a mixture of at least two

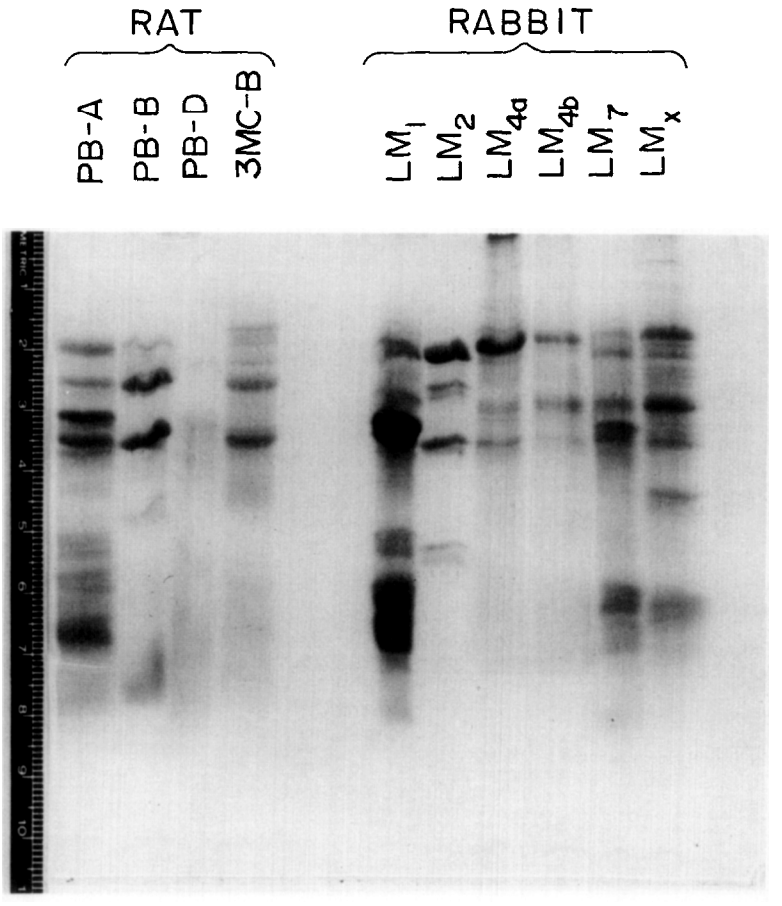

Figure 2. Peptide maps of P-450s after digestion with α-chymotrypsin. Experimental design was as in Fig. 1.

proteins, comparison of the digests with those obtained from other preparations is more difficult. Many of the bands derived from P-450 PB-B are also observed in the PB-A profiles; however, certain PB-B peptides do not appear in the PB-A profiles (Figs. 1,2), in support of the previous conclusion that these preparations contain different P-450s (8,16).

Rabbit liver P-450s LM_2 and LM_x, derived from PB-treated animals, are clearly dissimilar to each other and to other P-450s. P-450s LM_1 and LM_7, both isolated from untreated rabbits, differ in subunit molecular weights by 13,000 daltons (8,9) but show similar substrate specificity profiles

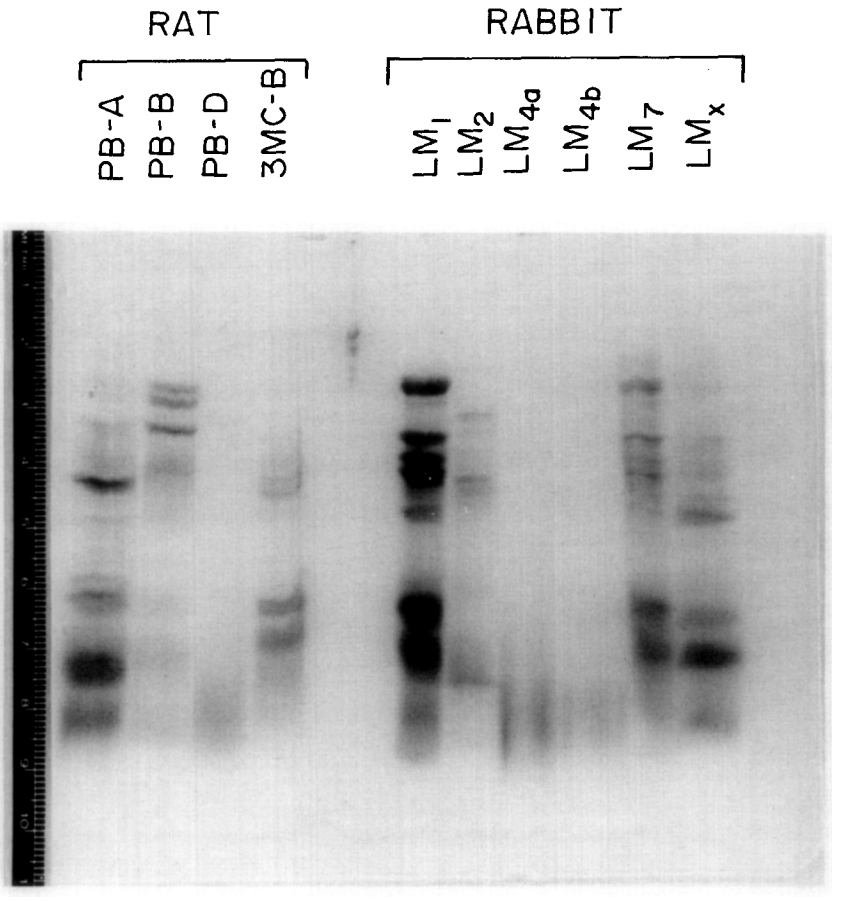

Figure 3. Peptide maps of P-450s after digestion with papain. Experimental design was as in Fig. 1.

(8); peptide maps of LM_1 and LM_7 clearly differ from those of other P-450s but do not differ appreciably from each other. Similarly, the P-450s LM_{4a} and LM_{4b} are not distinguished from each other by peptide mapping--these P-450s were both obtained from β-naphthoflavone-treated rabbits and have identical subunit molecular weights but differ in ability to metabolize certain substrates (8). P-450s LM_{4a}, LM_{4b}, and LM_x all have similar subunit molecular weights (8) but LM_x clearly differs in the peptide maps. Rat PB-B and rabbit LM_2 are the predominant P-450s induced by PB and have similar substrate specificities, but are readily distinguished by peptide mapping as

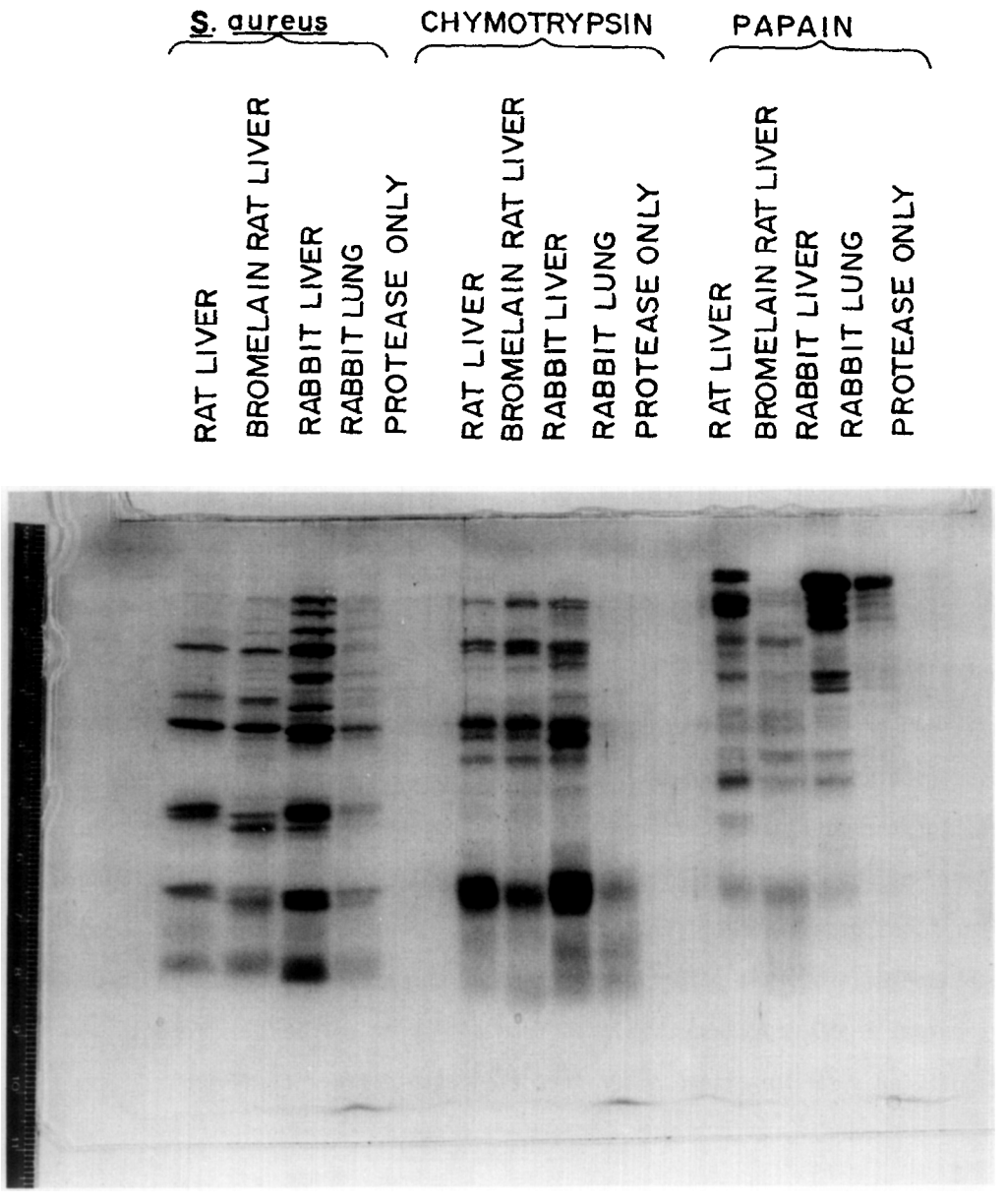

Figure 4. Peptide maps of NADPH-cytochrome P-450 reductases.

well as immunological cross-reactivity (24). Caution should be used in interpreting results obtained with the rabbit P-450s: all of the preparations are homogeneous as judged by SDS-polyacrylamide gel electrophoresis,

but further evidence for homogeneity has been obtained only for LM_2 (12,25).

The NADPH-cytochrome P-450 reductases have rather similar peptide maps, with some variation (Fig. 4). As expected, all bands in the bromelain-solubilized rat reductase preparation are present in the native reductase (detergent-solubilized). (The bromelain derivative, non-functional towards P-450, has a molecular weight 8,000 daltons less than does the native enzyme (21).) The inequality of the rat and rabbit liver reductases is most apparent in the papain digests; the rabbit liver and lung reductases [interchangeable in P-450 activity (11)] are similar although the lung enzyme is more susceptible to chymotrypsin. (The three native reductases have identical molecular weights (11).)

In conclusion, peptide mapping has been used to demonstrate major differences in highly-purified liver microsomal P-450s. However, significant similarity was found between certain rabbit P-450s which appear to be different proteins as judged by other techniques; the NADPH-cytochrome P-450 reductases are also rather similar. The results show that various P-450s are, for the most part, different proteins and that differences in physical properties and immunological and substrate specificity are not due to minor alterations of a basic polypeptide structure. Finally, this peptide mapping technique offers great potential for further comparison of P-450s and NADPH-cytochrome P-450 reductases isolated from different species, different tissues, or a single tissue after induction with different chemicals.

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REFERENCES

1. Lu, A.Y.H., Junk, K.W., and Coon, M.J. (1969) J. Biol. Chem. 244, 3714-3721.
2. Conney, A.H. (1967) Pharmacol. Rev. 19, 317-366.
3. Mannering, G.J. (1972) in Fundamentals of Drug Metabolism and Disposition (LaDu, B.N., Mandel, H.G., and Way, E.L., eds.), pp. 206-252, Williams and Watkins, Baltimore.
4. Welton, A.F., and Aust, S.D. (1974) Biochem. Biophys. Res. Commun. 56, 898-906.

5. Gielen, J.E., Goujon, F.M., and Nebert, D.W. (1972) J. Biol. Chem. 247, 1125-1137.
6. Thomas, P.E., Lu, A.Y.H., West, S.B., Ryan, D., Miwa, G.T., and Levin, W. (1977) Mol. Pharmacol. 13, 819-831.
7. Welton, A.F., O'Neal, F.O., Chaney, L.C., and Aust, S.D. (1975) J. Biol. Chem. 250, 5631-5639.
8. Guengerich, F.P. (1977) J. Biol. Chem. 252, 3970-3979.
9. Haugen, D.A., van der Hoeven, T.A., and Coon, M.J. (1975) J. Biol. Chem. 250, 3567-3570.
10. Ryan, D., Lu, A.Y.H., West, S., and Levin, W. (1975) J. Biol. Chem. 250, 2157-2163.
11. Guengerich, F.P. (1977) Mol. Pharmacol. 13, 911-923.
12. Haugen, D.A., and Coon, M.J. (1976) J. Biol. Chem. 251, 7929-7939.
13. Dean, W.L., and Coon, M.J. (1977) J. Biol. Chem. 252, 3255-3261.
14. Thomas, P.E., Lu, A.Y.H., Ryan, D., West, S.B., Kawalek, J., and Levin, W. (1976) Mol. Pharmacol. 12, 746-758.
15. Coon, M.J., Vermilion, J.L., Vatsis, K.P., French, J.S., Dean, W.L., and Haugen, D.A. (1977) in Drug Metabolism Concepts, (Jerina, D.M., ed.), pp. 46-71, Amer. Chem. Soc., Washington, D.C.
16. Guengerich, F.P., submitted.
17. Laemmli, U.K. (1970) Nature 227, 680-685.
18. Weber, K., Pringle, J.R., and Osborn, M. (1972) Methods Enzymol. 26, 3-27.
19. Neville, D.M., Jr. (1971) J. Biol. Chem. 246, 6328-6334.
20. Fairbanks, G., and Avruch, J. (1972) J. Supramol. Struct. 1, 66-75.
21. Welton, A.F., Pederson, T.C., Buege, J.A., and Aust, S.D. (1973) Biochem. Biophys. Res. Commun. 54, 161-167.
22. Cleveland, D.W., Fischer, S.G., Kirschner, M.W., and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102-1106.
23. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
24. Kamataki, T., Belcher, D.H., and Neal, R.A. (1976) Mol. Pharmacol. 12, 921-932.
25. Haugen, D.A., Armes, L.G., Yasunobu, K.T., and Coon, M.J. (1977) Biochem. Biophys. Res. Commun. 77, 967-973.