Characterization of a Major Form of Rat Hepatic Microsomal Cytochrome P-450 Induced by Isoniazid*

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Cytochrome P-450j has been purified to electrophoretic homogeneity from isoniazid-treated adult male rats; and this enzyme appears to be a major protein induced in hepatic microsomes after administration of isoniazid, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The hemoprotein has a minimum molecular weight of approximately 51,500, and the ferrous-carbonyl complex of cytochrome P-450j has a Soret maximum at 451-452 nm. The oxidized heme iron appears to be predominately in the high spin state as deduced from the Soret maximum at 395 nm. Ethylisocyanide binds to ferrous cytochrome P-450j to yield spectral maxima at approximately 458 and 430 nm with a resultant 458:430 ratio of 0.7 at pH 7.4. Cytochrome P-450j has no measurable catalytic activity for the metabolism of benz[a]pyrene (3- and 9-hydroxylation), hexobarbital, testosterone, and 5α-androstane-3α,17β-diol-3,17-disulfate. Low, but detectable, catalytic activity is obtained for the metabolism of 7-ethoxycoumarin, benzphetamine, p-nitroanisole, zoxazolamine, and 2-hydroxylation of 17β-estradiol. In contrast, cytochrome P-450j effectively catalyzes p-hydroxylation of aniline with a turnover of 12.7 nmol/min/nmol cytochrome P-450j. Hydroxyl radical scavengers, Fe-EDTA, superoxide dismutase, and catalase have no effect on aniline p-hydroxylation catalyzed by cytochrome P-450j.

Cytochrome P-450j is distinct from nine other rat hepatic microsomal cytochromes P-450 (P-450a-P-450i) previously purified in this laboratory, as well as different isozymes described by other investigators, based on several parameters including minimum molecular weight, spectral properties, and catalytic activity. In Ouchterlony double diffusion plates, antibodies against cytochromes P-450a-P-450f show no cross-reaction with cytochrome P-450j. Structural differences among cytochromes P-450a-P-450j are apparent from the NH2-terminal sequence of cytochrome P-450j, as well as the electrophoretic profiles of proteolytic digests of the hemoproteins.

Isoniazid (isonicotinic hydrazide) is a primary drug used in the treatment of tuberculosis; the major pathway for metabolism of the compound in man is acetylation to acetylsalicylic acid followed by hydrolysis to isonicotinic acid and acetylsalicylic acid (1-3). Enhanced metabolism of the ether anesthetic enflurane, as reflected in high plasma levels of inorganic fluoride, has been observed in some surgical patients who had been treated chronically with isoniazid (4). Hepatic microsomes from adult male rats administered isoniazid exhibit markedly increased rates of defluorination of enflurane, methoxyflurane, sevoflurane, and isofluorane compared to metabolism by microsomes from control rats (5, 6). An induction of microsomal anesthetic defluorination has also been reported for male rats treated with acetylsalicylic acid or hydrazine sulfate but not isonicotinic acid (7).

Rice and Talcott (5) have compared the catalytic activities of hepatic microsomes from rats treated with isoniazid, phenobarbital, or β-naphthoflavone toward a variety of substrates. The pattern of metabolism associated with isoniazid treatment did not resemble that of either phenobarbital or β-naphthoflavone induction. Specifically, the administration of isoniazid to male rats resulted in increased rates of metabolism of aniline, p-nitroanisole, ethoxyresorufin, and four other anesthetics (enflurane, methoxyflurane, sevoflurane, and isofluorane) and a depression of aminopine demethylation relative to control animals. Although no overall increase in microsomal cytochrome P-450 content was observed, isoniazid treatment resulted in an upward shift in the Soret maximum of the CO-reduced difference spectrum to 451 nm. Based on these findings, these investigators (5) proposed that isoniazid is a "new" class of inducer unlike phenobarbital or β-naphthoflavone.

This report describes the purification and characterization of cytochrome P-450j from isoniazid-treated adult male rats. Based on results of SDS1-polyacrylamide gel electrophoresis, this hemoprotein is apparently a major protein induced in hepatic microsomes after treatment of rats with isoniazid. Several properties of cytochrome P-450j clearly distinguish this enzyme from nine other rat cytochrome P-450 isozymes (P450a-P450i) previously purified (8, 9) in this laboratory. The results of this study indicate that isoniazid may be a "unique" inducer of cytochrome P-450 in the rat.

EXPERIMENTAL PROCEDURES

Purification of Cytochrome P-450—Forty adult male Long Evans rats (Blue Spruce Farms, Altamont, NY) at 8 weeks of age were

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; DTT, dithiothreitol.
administered ionized (Aldrich) in drinking water for 10 days. The drinking water was composed of 0.1% (w/v) isonicotinic acid hydrazide adjusted to pH 7.4, and the animals were allowed free access to Ralston Purina Rodent Chow 5001®. The rats were killed by decapitation, and hepatic microsomes were prepared as reported (10). Mitochondria were isolated from liver, washed with a buffer containing 8 g of protein, and the specific content of the preparations was approximately 1.0 nmol of cytochrome P-450/mg of protein. Except for the CM-Sepharose and phosphocellulose columns, the following purification procedure was conducted at 4°C.

A portion of a microsomal preparation containing 2 g of protein (—2000 nmol of cytochrome P-450) was diluted to 500 ml (4 mg protein/ml) in a buffer mixture to yield the following final concentrations: 0.1 M potassium phosphate buffer (pH 7.25), 20% glycerol (v/v), 1.0 mM EDTA, and 1.0 mM DTT. Sodium cholate, which had been recrystallized from ethanol, was added to the microsomal suspension to a final concentration of 0.6% (w/v). Recrystallized sodium cholate was used in all buffers. The mixture was stirred for 20 min, divided in half, and each portion was applied to an n-octylamine-Sepharose 4B column (2.2 × 25 cm). 1,8-Diaminooctane had been coupled to cyanogen bromide-activated Sepharose 4B as previously reported (11–13). Before sample application, the columns were each washed with 200 ml of 0.1 M potassium phosphate buffer (pH 7.25) and equilibrated with 160 ml of the same buffer containing 20% glycerol, 1.0 mM EDTA, 1.0 mM DTT, and 0.6% sodium cholate. The samples were applied, and each column was washed with 150 ml of 0.01 M potassium phosphate buffer (pH 7.25) containing 20% glycerol, 1.0 mM EDTA, 1.0 mM DTT, and 0.6% sodium cholate. The flow rate of the column was maintained at 1.1 ml/min. Approximately 15% of the total cytochrome P-450 eluted to the column was recovered in the final elution.

Based on SDS-polyacrylamide gel patterns, the fractions containing cytochrome P-450 were pooled and applied to a hydroxylapatite column (Hypatite C; Clarkson Chemical Co., Williamsport, PA) preswelled with 0.1 M potassium phosphate buffer (pH 7.25) containing 20% glycerol and 0.2% Emulgen 911. The column (2.2 × 10 cm) was washed with 40 ml of equilibration buffer, 300 ml of 0.02 M buffer with the same components, and was eluted with 125 ml of 0.25 M potassium phosphate buffer (pH 7.25) containing 20% glycerol and 0.2% Emulgen 911. The recovery of total microsomal cytochrome P-450 at this step was approximately 13%. The fractions containing cytochrome P-450j were pooled based on SDS-polyacrylamide gel profiles. The partially purified cytochrome P-450j preparation was concentrated by 500 ml of 0.4 M potassium phosphate buffer (pH 7.7) containing 20% glycerol, 1.0 mM EDTA, and 0.2% Emulgen 911. The cytochrome P-450j eluted from the column was maintained at 1.1 ml/min. Approximately 15% of the total cytochrome P-450j applied to the column was recovered in the final elution.

Purification of Other Rat Hepatic Microsomal Enzymes—Cytochromes P-450a-P-450i were purified to apparent homogeneity as previously reported (8, 9). NADPH-cytochrome c reductase was purified to a specific activity of 35,000–40,000 units/mg protein by a combination of the methods of Dignam and Strobel (14) and Yasukochi and Masters (15). Enzyme activity was assayed according to the method of Phillips and Langdon (16). One unit of reductase is defined as that amount catalyzing the reduction of 1 nmol of cytochrome c/min at 22°C. Electrophoretically homogeneous rat hepatic microsomal epoxide hydrolase was isolated as previously described (17).

Other Methods—Protein was determined by the method of Lowry et al. (18) with crystalline bovine serum albumin as standard. Cytochrome c reductase activity was determined according to the method of Omura and Sato (19) from the CO-reduced difference spectrum based on an extinction coefficient of 91 mM⁻¹ cm⁻¹. The binding of ethylisonic acid (2.0 mM final concentration) to ferrous cytochrome P-450 was measured as previously reported (20). The hemoprotein concentrations and buffer components used for spectral determinations are detailed in the appropriate figure legend. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (21) in a separating gel (7.5% acrylamide) 0.75-mm thick and 10-cm long. The gels were stained with Coomassie Blue R-250 and destained as described (17). Limited proteolytic digestion of the 10 purified hemoproteins in the presence of SDS was conducted as described by Cleveland et al. (22). The cytochromes P-450 were incubated with the protease for 10 min at 37°C at protein concentrations and buffer components used for spectral determinations were detailed in the appropriate figure legend. SDS-polyacrylamide gel electrophoresis in gels containing 12.5% acrylamide that were 1.5-mm thick and 10-cm long. The amino acid composition of cytochrome P-450j was analyzed by a previously described protocol (23). The NH₂-terminal sequence of the acetone-precipitated protein was determined (23) by manual Edman degradation.

Antibodies to purified cytochromes P-450-P-450f were produced and purified as previously described (24–26). Ouchterlony double diffusion plates were prepared as described (25).

Catalytic activity of cytochrome P-450 was assayed under conditions in which metabolism was proportional to hemoprotein concentration and time of incubation. In each experiment, other purified cytochrome P-450 isoforms with known activity were included for reference. Saturating amounts of NADPH-cytochrome c reductase and optimal diilauroylphosphatidylcholine were used in all experiments. Diilauroylphosphatidylcholine was prepared in water and sonicated immediately before use. For the following reference contain the methods for assays of the various substrates: [N-methyl-¹⁴C]benzphetamine (27), [2-⁴C]hexobarbital (28, 29), benz[α]pyrene (30), [⁴H]xoxazolamidine (31), [²H]estradiol-17β (32), 7-ethoxycoumarin (33), and p-nitroanisole (34). Metabolites of testosterone were analyzed by reverse phase high pressure liquid chromatography (35, 36).
RESULTS

Purification of Cytochrome P-450j—Cytochrome P-450j was purified from hepatic microsomes of isoniazid-treated adult male rats by chromatography on n-octylamine-Sepharose 4B, hydroxylapatite, DEAE-Sepharose, CM-Sepharose, and phosphocellulose, as detailed under “Experimental Procedures,” with a yield of approximately 2% of total microsomal cytochrome P-450. The initial n-octylamine-Sepharose 4B column was based on the original approach of Imai and Sato (41) for the purification of rabbit liver cytochrome P-450 as modified by Guengerich et al. (13, 42) for rat liver enzymes but utilized buffer mixtures that were most effective for the purification of cytochrome P-450j. A dramatic purification of cytochrome P-450j is achieved by chromatography on n-octylamine-Sepharose 4B; based on SDS-polyacrylamide gels, only 4–5 major contaminating proteins co-elute with this enzyme. The subsequent hydroxylapatite column effects a removal of two major contaminants from the partially purified cytochrome P-450j preparation. The third step in the procedure, the DEAE-Sepharose column, was the most difficult to develop since cytochrome P-450j does not bind well to most anionic exchange resins under several conditions. Optimal recovery and purification of the hemoprotein are achieved in the presence of a high concentration of Emulgen 911 (0.6%). If this step is eliminated from the procedure, the CM-Sepharose column is ineffective at removing certain remaining contaminants. As judged by SDS-polyacrylamide gel patterns, these contaminating proteins are removed from the cytochrome P-450j preparation by chromatography on CM-Sepharose if preceded by the DEAE-Sepharose column. Residual trace contaminants are eliminated from the cytochrome P-450j preparation by chromatography on phosphocellulose.

Fig. 1 shows an SDS-polyacrylamide slab gel (0.75-mm thick) of the purified hemoprotein that illustrates the apparent homogeneity of the enzyme preparation even at high protein concentrations. Only one protein-staining band corresponding to cytochrome P-450j is observed; however, at high concentrations, a very trace contaminant of low minimum Mr is detected in some enzyme preparations. The minimum Mr of cytochrome P-450j was determined to be 51,500 in the gel system of Laemmli (21), based on the electrophoretic mobility of the protein relative to cytochromes P-450a–P450i, epoxide hydrolase, and NADPH-cytochrome c reductase as shown in Fig. 1. The distinct mobility of cytochrome P-450j is apparent when a comparison is made between the mobilities of the other purified microsomal enzymes in the first well (labeled MIX), and cytochrome P-450j. Therefore, cytochrome P-450j is distinct from the nine other purified rat hepatic microsomal hemoproteins (cytochromes P-450a–P450i) previously purified in our laboratory (8, 9) based on minimum Mr. As shown in the last well (labeled MIX), however, when cytochrome P-450j is included in the mixture of proteins, cytochromes P-450b (Mr, 52,000), P-450j, and P-450d/P-450h (Mr, 51,000) appear as a single broad protein-staining band. In contrast to the report of Guengerich et al. (42), the relative mobilities of the 10 cytochromes P-450 relative to each other were unaffected by the substitution of lithium dodecyl sulfate for SDS in this gel system (data not shown). Results of preliminary experiments (data not shown) suggest that cytochrome P-450j, like cytochromes P-450c and P-450d (43), tends to streak in two-dimensional isoelectric focusing-SDS gel electrophoresis.

Fig. 2 shows the electrophoretic profiles of purified cytochrome P-450j and hepatic microsomes from untreated and isoniazid-treated adult male rats in two SDS-polyacrylamide slab gels. In the experiment on the left, electrophoresis was stopped when the tracking dye reached the bottom of the gel; in the experiment on the right, electrophoresis was continued for an additional 30 min. The samples and all other conditions were identical for the two experiments. As illustrated on the left, the electrophoretic patterns of hepatic microsomes from untreated and isoniazid-treated rats are indistinguishable except for a marked broadening of a protein-staining band at ~51,000 in the profile from the treated animals. The upper region of this broadened band has the same electrophoretic mobility as purified cytochrome P-450j. When electrophoresis was continued for a longer period of time, as shown on the right, the broadened protein-staining band can be resolved into two bands. Purified cytochrome P-450j co-migrates with the higher Mr band. Hepatic microsomes from untreated rats appear to have only a trace amount of the higher Mr protein as evidenced by the lack of a distinct protein-staining band of that mobility. Within the inherent limitations to the interpretation of SDS-polyacrylamide gel profiles of hepatic microsomes, these observations suggest that isoniazid is a selective inducer of a protein(s) with a minimum Mr, corresponding to cytochrome P-450j and that hepatic microsomes from untreated rats contain very little of that protein. Interestingly, as originally reported by Rice and Talcott (5), no increase in...
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Fig. 2. SDS-polyacrylamide slab gels of rat hepatic microsomes and cytochrome P-450j. The purified enzyme was applied to each of the wells labeled "j" at 0.3 μg. Hepatic microsomes (6 μg) from untreated and isoniazid-treated adult male rats were applied to the wells designated UN and ISN, respectively. The protocol for both slab gels shown was identical except electrophoresis was stopped when the tracking dye (bromphenol blue) reached the bottom of the gel in the experiment on the left, whereas electrophoresis was continued for an additional 30 min in the experiment on the right.

Fig. 3. Spectral characteristics of purified cytochrome P-450j. A, CO- and ethylisocyanide difference spectra of ferrous cytochrome P-450j. The CO-reduced difference spectrum was recorded at a hemoprotein concentration of 1.4 nmol/ml in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 0.2% Emulgen 911, and 0.5% sodium cholate. The purified enzyme was diluted to 0.8 nmol/ml in the same buffer mixture for the generation of the ethylisocyanide difference spectrum. A final concentration of 2.0 mM ethylisocyanide was added to the sample cuvette containing ferrous cytochrome P-450j, and the difference spectrum was recorded. B, absolute spectra of cytochrome P-450j. The sample cuvette contained cytochrome P-450j (1.2 nmol/ml) diluted in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.2% Emulgen 911, and 0.5% sodium cholate, and the reference cuvette contained the same buffer mixture.

Total cytochrome P-450 content occurs when rats are treated with isoniazid. Therefore, one might predict that an induction of cytochrome P-450j occurs with a concomitant decrease in another form(s) of cytochrome P-450. However, no marked decrease in any protein-stained band in the electrophoretic pattern of microsomes form isoniazid-treated rats relative to the protein profile of microsomes from untreated animals is observed in the M region of cytochrome P-450 (45,000-60,000).

Spectral Properties—Fig. 3 illustrates the CO- and ethylisocyanide-difference spectra of ferrous cytochrome P-450j (A) as well as the absolute spectral characteristics of the purified hemoprotein (B). The protein was diluted in a buffer mixture containing Emulgen 911 and sodium cholate for all spectral measurements, since detergents have a protective influence on the enzyme. The CO-reduced difference spectral maximum of the enzyme is at approximately 451-452 nm, and the absence of a shoulder at 420 nm indicates that the cytochrome P-450j preparation does not contain a significant amount of cytochrome P-420 (Fig. 3A). Rice and Talcott (5) had reported that isoniazid treatment of rats resulted in an upward shift in the hepatic microsomal CO-reduced difference spectral maximum to 451 from 450 nm observed in the spectrum from untreated animals. This shift is most likely the consequence, at least in part, of the induction of cytochrome P-450j by isoniazid. Of the other rat cytochromes P-450 previously purified in our laboratory (8, 9), the Soret maximum of the ferrous carboxyl complex of five show a shift downward to 447–449 nm (cytochromes P-450c, P-450d, P-450f, P-450g, P-450i), one is at 450 nm (cytochrome P-450h), and three are shifted upward to 451–452 nm (cytochromes P-450a, P-450e, P-450j).

Two spectral maxima are generated at 458 and 430 nm when the ethylisocyanide difference spectrum of ferrous cytochrome P-450j is recorded at pH 7.4, as shown in Fig. 3A. The 458/
430 peak ratio at pH 7.4 was calculated to be 0.7 which is similar to the ratio observed in the ethylisocyanide difference spectrum of ferrous cytochrome P-450b (8). The spectrum of ferrous cytochrome P-450j bound with ethylisocyanide is distinct from the corresponding spectra of cytochromes P-450a-P-450i in that an absorption peak is observed at 438 nm. A Soret maximum at 452-455 nm is observed in the ethylisocyanide difference spectra of the nine other hemoproteins. A peak or shoulder at 430 nm is characteristic of the spectrum of all of these hemoproteins. The ethylisocyanide difference spectral characteristics of cytochromes P-450a-P-450i have been previously described (8, 9). Unlike cytochromes P-450b, P-450c, P-450g, and P-450i, the ligand metyrapone does not bind to ferrous cytochrome P-450j (data not shown).

The absolute spectral properties of cytochrome P-450j are shown in Fig. 3B. Cytochrome P-450j is primarily a high spin ferric hemoprotein as indicated by the absorption maximum at 395 nm in the absolute oxidized spectrum of the enzyme (E = 90 mM⁻¹ cm⁻¹). The extinction coefficient and absorption maximum (395 nm) of oxidized cytochrome P-450j is not influenced by the addition of detergents or glycerol or affected by protein concentration from 0.5–6.0 μM. Cytochrome P-450d is a high spin ferric hemoprotein, cytochrome P-450f is predominately high spin, and cytochrome P-450e contains a relatively minor high spin component, whereas cytochromes P-450a, P-450b, P-450c, P-450g, P-450h and P-450i are low spin ferric hemoproteins as determined by their absolute oxidized spectral characteristics (8, 9, 17). When the hemoprotein is reduced with sodium dithionite, the Soret maximum of cytochrome P-450j shifts downward to approximately 412 nm. The CO-reduced absolute spectral maximum of cytochrome P-450j is at 452 nm.

**Fig. 4. Ouchterlony double diffusion analysis.** Antibodies to cytochromes P-450a–P-450f were prepared and purified as previously reported (24–26). The components of the immunodiffusion plate have been described elsewhere (25). Purified cytochromes P-450 (center well), P-450b and P-450c (bottom well) were each present at 4 μM. Antibodies to cytochromes P-450a–P-450f at 25 mg/ml were applied to the peripheral wells, as indicated.

**Immunoochemical Reactivities—Ouchterlony double diffusion analysis** was used to detect potential reactivity of cytochrome P-450j with antibodies prepared against cytochromes P-450a–P-450f with the results shown in Fig. 4. There is no detectable recognition of cytochrome P-450j by these antibodies. Although the Ouchterlony plate shown in Fig. 4 contained 0.2% Emulgen, the same results were obtained in the absence of detergent (data not shown). Although antibody to cytochrome P-450e was not included in the immunodiffusion plate, both cytochromes P-450b and P-450e are immunologically indistinguishable (8) when tested against polyclonal antibody to cytochrome P-450b. Therefore, cytochrome P-450j is immunologically distinct from cytochromes P-450a–P-450f previously purified in our laboratory. Antibody prepared against cytochrome P-450f cross-reacts strongly with the heterologous proteins, cytochromes P-450g, P-450h, and P-450i. Since cytochrome P-450j was not recognized by antibody to cytochrome P-450f, cytochrome P-450j also differs immunologically from cytochromes P-450g, P-450h, and P-450i. Preliminary results also indicated that cytochrome P-450j does not react with several monoclonal antibodies (44) to cytochrome P-450c in an enzyme-linked immunosorbent assay.

**Peptide Mapping—**Fig. 5 shows the comparative SDS-polyacrylamide gel profiles of cytochromes P-450a–P-450j after limited proteolysis in the presence of SDS by the method of Cleveland et al. (22). The peptides of each hemoprotein generated by cleavage with chymotrypsin (Fig. 5A) or Staphylococcus aureus V8 protease (Fig. 5B) under identical conditions were analyzed electrophoretically in a SDS-polyacrylamide slab gel (1.5-mm thick) that was composed of 12.5% acrylamide. The wells are labeled by the subscript of the cytochrome P-450, and each protease alone was applied to the gel as indicated. As illustrated in Fig. 5A, cytochrome P-450j is relatively resistant to digestion by chymotrypsin compared to most of the other isozymes. The peptide map of cytochrome P-450j contains two major peptides of relatively large minimum Mₐ as well as the undigested hemoprotein at a Mₐ of 51,500. The chymotryptic digest of cytochrome P-450j is markedly different from the peptides generated by cleavage of cytochromes P-450a–P-450i.

Fig. 5B shows the gel patterns of peptides of cytochromes P-450a–P-450j after digestion with S. aureus V8 protease which cleaves adjacent to aspartic and glutamic acid residues. The protease cleaves cytochrome P-450j at several sites yielding a peptide map clearly distinguishable from the maps of cytochromes P-450a–P-450i. The results presented in Fig. 5 indicate that cytochrome P-450j differs structurally from cytochromes P-450a–P-450i.

**Amino Acid Composition and Amino-terminal Sequence—**The amino acid content of cytochrome P-450j was analyzed with the results shown in Table I. The composition of the hemoprotein is similar to those of cytochromes P-450a–P-450i (23) since all of these enzymes are composed of 40–50% hydrophobic residues (Pro, Ala, Val, Met, Ile, Leu, Phe) although significant differences in content among the cytochromes are also apparent. Cytochrome P-450j contains five Cys residues/molecule which is fewer than any of the other proteins except cytochrome P-450a (23).

The NH₂-terminal sequence of cytochrome P-450j, determined by Edman degradation for the first 19 residues, is also listed in Table I. The purity of the hemoprotein preparation was confirmed by the presence of a single NH₂-terminal sequence. The NH₂-terminal residue of cytochrome P-450j is Ala, and the enzyme possesses a hydrophobic leader sequence; 15 of the first 19 amino acid residues are hydrophobic. Very little, if any, homology is observed in the NH₂-terminal se-
Electrophoretic patterns of 10 purified rat hepatic cytochromes P-450 generated by proteolytic digestion. In parts A and B, each hemoprotein was subjected to limited proteolytic digestion under identical conditions according to the protocol of Cleveland et al. (22). The cytochromes P-450 were incubated with chymotrypsin (0.05 mg/mg of hemoprotein) as shown in part A or S. aureus V8 protease (0.15 mg/mg of hemoprotein) as shown in part B, for 10 min at 37 °C. The wells are labeled by the subscript of the cytochrome and contain 2.8 pg of each digest. Chymotrypsin (CHT) was applied to the first and last wells in part A, and S. aureus V8 protease (V8) was applied to the last well of the gel in part B.

Table I
Amino acid composition and amino-terminal sequence of cytochrome P-450j

| Amino acid | Residues/molecule | NH2-terminal sequence |
|------------|-------------------|----------------------|
| Asp        | 44                | 1 Ala (168)          |
| Thr        | 22                | 2 Val (205)          |
| Ser        | 23                | 3 Leu (262)          |
| Glx        | 45                | 4 Gly (138)          |
| Pro        | 31                | 5 Ile (131)          |
| Gly        | 30                | 6 Thr (60)           |
| Ala        | 22                | 7 Ile (61)           |
| Val        | 33                | 8 Ala (137)          |
| Met        | 8                 | 9 Leu (124)          |
| Ile        | 31                | 10 Leu (118)         |
| Leu        | 61                | 11 Val (121)         |
| Tyr        | 15                | 12 Trp (45)          |
| Phe        | 30                | 13 Val (85)          |
| Lys        | 36                | 14 Ala (77)          |
| His        | 9                 | 15 Thr (23)          |
| Arg        | 22                | 16 Leu (100)         |
| Trp        | ND                | 17 Leu (136)         |
| Cys        | 5                 | 18 Val (90)          |

M, 52,000

Catalytic Activity—Table II lists the catalytic activity of purified rat hepatic cytochrome P-450j toward several substrates and various types of reactions. The hemoprotein has no measurable activity toward the metabolism of hexobarbital or benz[a]pyrene nor does it participate in the hydroxylation of the steroids, testosterone, or 5α-androstane-3a,17β-diol-3,17-disulfate. Low but measurable metabolism of benzphetamine, zoxazolamine, 7-ethoxyxoumarin, estradiol-17β (2-hydroxylation), and p-nitroanisole is catalyzed by cytochrome P-450. The

Table II
Catalytic activity of purified rat hepatic cytochrome P-450j

| Substrate               | Reaction         | Turnover number |
|-------------------------|------------------|-----------------|
| N-Demethylation         | Benzphetamine    | 5.5             |
| 3-Hydroxylation         | Hexobarbital     | <0.5            |
| 6-Hydroxylation         | Benzo[a]pyrene   | <0.1            |
| 2-Hydroxylation         | Zoxazolamine     | 3.4             |
| 2-Hydroxylation         | Estradiol-17β    | 0.8             |
| O-deethylation          | 7-Ethoxyxoumarin | 1.2             |
| O-demethylation         | p-Nitroanisole   | 1.6             |
| Hydroxylation           | Testosterone      | <0.1            |
| Hydroxylation           | Androstane disulfate | <0.1   |
| Hydroxylation           | Aniline          | 12.7            |

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addition of cytochrome b₅ to the reconstituted system at 0.8 nmol/nmol cytochrome P-450j has no effect on the O-de-methylation of p-nitroanisole (data not shown). In contrast to the results with the other substrates, cytochrome P-450j effectively catalyzes p-hydroxylation of aniline with a turnover of 12.7 nmol/min/nmol cytochrome P-450. Of the iso-zymes purified in our laboratory, cytochrome P-450j has the highest catalytic activity toward the metabolism of aniline. Cytochrome P-450j is a relatively effective catalyst of aniline p-hydroxylation (9.6 nmol/min/nmol cytochrome P-450j); cytochromes P-450b, P-450c, and P-450h have low but measurable activity (1.0-2.0 nmol/min/nmol cytochrome P-450j), whereas cytochromes P-450a, P-450e, P-450f, P-450g, and P-450i are very poor catalysts of this reaction (<0.5 nmol/min/nmol cytochrome P-450j). Therefore, the substrate selectivity of cytochrome P-450j is clearly distinct from the enzymatic activities of cytochromes P-450a-P-450i (8, 9, 38).

One catalytic parameter that was used by Rice and Talcott (5) to determine if isoniazid is a “unique” inducer of rat hepatic microsomal cytochrome P-450 was aniline p-hydroxylation. A 3.5-fold induction of aniline hydroxylation, expressed per nmol of cytochrome P-450, was observed in microsomes from isoniazid-treated rats relative to control values. No increase (per nmol of cytochrome P-450) was detected after phenobarbital or β-naphthoflavone treatment of rats. The marked increase in microsomal metabolism of aniline after treatment of animals with isoniazid may be the consequence, at least in part, of the induction of cytochrome P-450j by this compound.

Influences of Effectors on p-Hydroxylation of Aniline—Table III summarizes the effects of hydroxyl radical scavengers (mannitol, dimethyl sulfoxide), iron chelators (EDTA, deferoxamine), superoxide dismutase and catalase, as well as Fe-EDTA on p-hydroxylation of aniline catalyzed by cytochromes P-450j and P-450d. The mechanism of this reaction was studied using cytochromes P-450j and P-450d which have the highest catalytic activities for this substrate of the rat hepatic isozymes purified in our laboratory. The results listed in Table III show that the rate of p-hydroxylation of aniline catalyzed by cytochromes P-450j or P-450d is unaffected by the addition of any of the effectors tested. The maximum effect of any addition is approximately 20% inhibition observed when Fe-EDTA (0.05 mM) is added to an incubation containing cytochrome P-450j as the catalyst. The results presented in Table III contrast with the proposed mechanism of aniline p-hydroxylation reported by Ingelman-Sundberg and Ekstrom (49).

In the study of Ingelman-Sundberg and Ekstrom (49), aniline p-hydroxylation catalyzed by purified rabbit liver cytochrome P-450 LM2 was reported to be mediated by hydroxyl radicals that were generated by an iron-catalyzed Haber-Weiss reaction between superoxide anions and hydrogen peroxide. The proposed mechanism was derived from results showing a marked inhibition of aniline hydroxylation in the presence of catalase, superoxide dismutase, and several hydroxyl radical scavengers. If the formation of hydroxyl radicals occurred via the Haber-Weiss scheme, the addition of Fe-EDTA would be expected to enhance the reaction, and deferoxamine should inhibit hydroxylation. As outlined in Table III, however, p-hydroxylation of aniline catalyzed by cytochrome P-450j or P-450d is not affected to any significant extent by any of the potential effectors tested. The reason for this discrepancy is unknown but may be related to the specific cytochrome P-450 used as the catalyst.

**Table III**

| Addition       | Percent control activity | % control activity |
|----------------|--------------------------|--------------------|
| None           | 100 (12.7)               | 100 (9.6)          |
| EDTA (0.1 mM)  | 105                      | 115                |
| Fe-EDTA (0.1 mM)| 81                       | 102                |
| Mannitol (100 mM)| 107                    | 102                |
| Dimethyl sulfoxide (50 mM)| 88                 | 97                 |
| Desferrioxamine (0.3 mM)| 85               | 88                 |
| Superoxide dismutase (280 units, 100 µg)| 97            | 101                |
| Catalase (200 units, 15 µg)| 94           | 104                |

DISCUSSION

Cytochrome P-450j has been purified from adult male rats and has been shown to be a distinct isozyme from nine other cytochromes P-450 (P-450a-P450) previously purified in our laboratory (8, 9) based on amino-terminal sequence analysis. Several biochemical properties of cytochrome P-450j are different from the other purified hemoproteins. Additionally, cytochrome P-450j is clearly distinguishable from several other rat hepatic microsomal cytochromes P-450 purified from untreated or induced animals by other investigators assessed on various characteristics of the purified enzyme. The NH₂-terminal sequence of cytochrome P-450j is different from the sequences of rat liver cytochromes P-450 PB-1 (45), PB-2 (46), RLM5 (47), and RLM3 (47). Of the hemoproteins purified by Guengerich and co-workers (42, 50), cytochrome P-450 BNF/[SF-6, which corresponds to cytochrome P-450d, has the highest catalytic activity toward p-hydroxylation of aniline and is the only hemoprotein exhibiting high spin characteristics in its absolute ferric spectrum. Therefore, cytochrome P-450j probably does not correspond to any of the hemoproteins purified in that laboratory. A composite of the spectral, electrophoretic, and catalytic properties of forms 1-5 isolated from β-naphthoflavone-treated rats (51), cytochrome P-452 from clofibrate-treated rats (52), and PCN cytochrome P-450 from pregnenolone 16α-carbonitrile-treated rats (53) indicate that none of these hemoproteins correspond to cytochrome P-450j. Nor do the properties of the rat liver microsomal cytochromes P-450 purified by Waxman and co-workers (45, 54, 55) resemble cytochrome P-450j. Cytochrome P-450 PB-1 (45) has a different NH₂-terminal sequence than cytochrome P-450j, whereas cytochromes P-450 PB-2c; PB-3; PB-4, and PB-5 (54, 55) correspond to cytochromes P-450h, P-450a, P-450b, and P-450e, respectively. These results support the original proposal of Rice and Talcott (5) that isoniazid in a “unique” inducer of cytochrome P-450 in the rat, unlike phenobarbital or β-naphthoflavone.

Over the past several years, our laboratory has been studying the regulation of rat liver cytochromes P-450 by immunochemical quantitation of levels of certain isozymes in microsomes from untreated rats as well as animals induced by numerous compounds (24, 25, 56). That structurally diverse compounds can induce the same cytochrome P-450 isozymes has become increasingly apparent. Interestingly, certain characeristics of microsomal preparations from rats treated with
ethanol, pyrazole, and acetone as well as diabert or fasted animals suggest that cytochrome P-450 may be induced by these treatments. Microsomal electrophoretic patterns indicate that ethanol treatment of rats results in the appearance of a protein-staining band (M = 51,500) of the same mobility as cytochrome P-450j (9, 57, 58). Several laboratories have shown hepatic microsomes from male rats treated with ethanol, pyrazole, and acetone as well as diabert (genic, alloxan-, or streptozotocin-induced) animals or fasted rats exhibit similar SDS-polyacrylamide gel electrophoretic profiles with an increase in a protein-staining band with a M, similar to cytochrome P-450j (57-64). Yang and co-workers (59, 63, 64) have reported a correlation between induction of a high affinity NADPH-dependent nitrosodimethylamine demethylation and the appearance of this protein-stained band in SDS-gel profiles. Diabetic rats and animals treated with certain of these compounds is increased levels of ketone bodies.

In the rabbit, cytochrome P-450 LM3a, an isozyme with high catalytic activity toward aniline p-hydroxylation as well as the oxidation of alcohols (40), is apparently inducible by ethanol, isoniazid, imidazole, pyrazole, trichloroethylene, and m-xylene as determined by an increase in microsomal aniline hydroxylation following treatment with these agents (70). In fact, Koop and Coon (71) have purified and characterized cytochrome P-450 LM3a from imidazole-treated rabbits and have shown that isozyme to be identical to the corresponding hemoprotein from ethanol-treated animals. The results of Ingelman-Sundberg and Johansson (72) suggest that benzene may also induce the same cytochrome(s) P-450 (LMeb) as cytochrome P-450 LM3a in rabbit liver. Studies are currently in progress to verify this proposal.

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