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CHARACTERIZATION OF HUMAN MICROSOMAL CYTOCHROME P-450 ENZYMES

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INTRODUCTION

Cytochrome P-450[1] enzymes catalyze the oxidation of a wide variety of drugs, pesticides, carcinogens, toxicants, pollutants, and ingested natural products as well as endogenous compounds such as steroids, fatty acids, fat-soluble vitamins, and eicosanoids (2, 3, 7). These reactions may be grouped into the categories of carbon hydroxylation, heteroatom oxygenation, heteroatom release, dehydrogenation, epoxidation, oxidative group transfer, and some other reactions that result in enzyme inactivation (3, 8). P-450s are also known to catalyze some reductive reactions. The oxidative reactions can be understood in the context of abstraction of hydrogen atoms or non-bonded or π electrons from the substrate by hypervalent iron (formally FeO^{3+}) and radical recombination. The reductive reactions, studied less exhaustively, involve electron transfer via a ferrous/ferric couple. The basic chemistry involved in the various reactions (oxidative or reductive) is probably relative-

[1]P-450, cytochrome P-450. In general, individual P-450 enzymes will be referred to by the nomenclature used in the author's own laboratory or else that used by others in cited literature in specific instances. For further reference to comparisons of preparations studied in different laboratories, the reader is directed to references (1–5). P-450 *genes* are tentatively classified according to the nomenclature of Nebert et al (6); several gaps still exist in the association of proteins and genes in cases of closely related sequences. The term "*isozymes*" will not be used in the context of discussing related P-450 enzymes; in its strict definition the term refers to enzymes that catalyze the same reaction, and most of the emphasis with P-450s is on the selective catalysis of different reactions.

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ly constant and the selectivity of different P-450s for different reactions is thought to be the result of distinctions in the three-dimensional binding sites of the enzymes for substrates and transition states. A single P-450 metabolizes a number of distinct reactions, if the proper juxtaposition of the molecule in the binding site occurs, and thus the broad substrate specificity of the total set of P-450s can be explained by the existence of a limited number of enzymes (20–30) within each animal species and tissue.

Much progress has been made in understanding of P-450 enzymes through studies with experimental animals and microorganisms. A number of reviews describe studies on the chemistry involved in catalysis (8–10), gene structure and regulation (11–13), and roles of individual enzymes in various reactions (2–5, 7, 14–16). All of this information has been very helpful in shaping the manner in which we think about the P-450 enzymes. However, there are limitations in the extrapolation of studies from experimental animal models to humans. These limitations have practical consequences in understanding drug disposition and risk assessment in man. One problem is the existence of sexual bimorphism in P-450 enzymes in rodents—although the biology is interesting, the situation is probably irrelevant in humans, for the observed pharmacokinetic differences between the sexes are relatively small (17). The various experimental animals differ among each other in the inducibility of P-450s—for instance, rats respond well to pregnenolone-16α-carbonitrile, but rabbits do not. Even within a species, individual strains may vary in the inducibility of P-450s (18–20). Thus, we see that even among very orthologous structural genes there is considerable variation in regulatory mechanisms. In addition, highly orthologous P-450s, coded for by closely-related structural genes, may differ markedly in catalytic specificity. For instance, mouse P_1-450 and rat P-450$_{\beta NF\text{-}B}$ are 98% identical in their sequences and catalyze many of the same reactions; however, only the latter protein catalyzes warfarin 6- or 8-hydroxylation (21). Human P-450$_{MP}$, a 4-mephenytoin 4-hydroxylase, shows considerable sequence similarity with certain rat, rabbit, and human proteins that have distinctively different catalytic activities (22, 23). Finally, experimental animals produce a somewhat biased view of variation in the human population, in that they are not so completely outbred and consume defined diets, which presents an artificial paradigm.

Fortunately, some recent developments have permitted more insight into the biochemical characterization of human P-450 enzymes and genes. First, the technical and conceptual advances made in the study of experimental animal systems have been helpful. In recent years access has been more available to human liver and other useful samples. The identification of polymorphisms in humans at the in vivo level has led to very selective assays for individual P-450 enzymes. Finally, the identification of human orthologs

of animal P-450s has been possible through the use of antibodies and oligonucleotide probes.

With this background, the current knowledge about individual human P-450 enzymes is presented. Only the microsomal P-450s are discussed; the two major mitochondrial enzymes, P-450scc (cholesterol side chain cleavage enzyme) and P-450 $_{11\beta}$ (11β-hydroxylase), are involved in relatively specific steroid hydroxylations. While neither protein has been purified from human tissue, the genes for both have been sequenced (24–26).

P-450$_{DB}$

Debrisoquine 4-hydroxylation was the first P-450-linked activity shown to be polymorphically distributed and under monogenic control (27). The same enzyme appears also to be responsible for the bulk of the oxidation of sparteine (28) and at least 20 other drugs (29, 30). The immunochemical similarity to a rat P-450 (P-450$_{UT\text{-}H}$) was used in the partial characterization (31) and isolation (32) of human P-450$_{DB}$. The enzyme appears to be a relatively minor form of human P-450 in terms of its total concentration in the liver, although in vitro studies indicate that it is the primary catalyst of debrisoquine 4-hydroxylation, sparteine Δ^2- and Δ^5-oxidation, (+) and (−) bufuralol 1'-hydroxylation, propranolol 4-hydroxylation, encainide O-demethylation, (+) and (−) metoprolol α-hydroxylation and O-demethylation, desmethylimipramine 2-hydroxylation, dextrometorphan O-demethylation, and nortryptilline 10-hydroxylation (31–35). Quinidine is a very effective inhibitor both in vitro (K_i ~60 nM) and in vivo (36), although the oxidation of quinidine is not catalyzed by P-450$_{DB}$ (37). Other corresponding proteins include P-450$_{Buf\ I}$ (33, 34) and P-450$_{NT}$ (35).

Recently Gonzalez et al (38) have sequenced both cDNA and genomic clones related to human P-450$_{DB}$. Three related gene-like sequences (P-450 II DI) occur in tandem on chromosome 22 (22qll.2-qter); only one is transcribed because the others (homologous pseudogenes) are interrupted with start and stop sequences. The catalytic activity found in different individuals is correlated with the amount of P-450$_{DB}$ detected with antibody raised to the rat liver homolog (31, 38) and the amount of a 1.8-kb form of mRNA (38). The gene product is catalytically active when expressed in COS kidney cells. Three different mutations have been found in individuals of the poor metabolizer phenotype—in each case mRNAs of abnormal length are produced, due to mutations in the genome outside of the protein coding region, that do not result in the production of stable proteins. Restriction fragment-length polymorphisms related to the three defects can be used to explain more than half of the poor metabolizer cases (39).

The existence of a single human gene contrasts with the presence of four genes (at least two of which can be expressed) in rat liver (40, 41). Since only

a single gene is expressed in humans, the existence of a second, related protein with distinct activity (34) must be questioned, with only posttranslational modification as a possible explanation. The existence of the second protein is also not compatible with quinidine inhibition studies (34, 37). The reports that catalytic activity is not correlated to the amount of protein detected with polyclonal antihuman P-450$_{DB}$ are probably the result of lack of specificity of the antibodies used (32, 34). The reports that liver microsomes isolated from poor metabolizers of debrisoquine showed similar V_{max} but much higher K_m values for sparteine Δ^2-oxidation (42) and (+) bufuralol 1'-hydroxylation (43) differ with the paradigm presented for abnormal mRNA splicing (39).

While several lines of evidence support the view of Gonzalez et al (38), a number of questions still exist about the nature of the variation in the oxidation of debrisoquine and related compounds. Although the defective mRNAs can explain many cases of the poor metabolizer phenotype, the variation within the extensive metabolizer phenotype is still considerable (about 100–fold as reflected in in vivo metabolic ratios). To date, the phenotypes have not sorted well into trimodal groups with heterozygotes clearly distinguished from other groups. Since only one gene is transcribed, the variation might be due to either structural alterations within the protein coding region (or possibly posttranslational?) or to the amount of P-450$_{DB}$ formed. Correlations may lend support to the latter view (31, 38). If this hypothesis is correct, then either variations in *cis*- or *trans*-acting regulatory elements may need to be invoked. At this time no clear evidence for induction of P-450$_{DB}$-related activity has been reported.

The basis of the reported segregation of phenacetin O-deethylation with debrisoquine 4-hydroxylation (44, 45) remains unexplained, since several lines of evidence now clearly indicate the involvement of different P-450s, coded for by different genes on different chromosomes and under independent regulation (see below). Also, debrisoquine 4-hydroxylation and sparteine Δ^2-oxidation do not cosegregate in some individuals (46, 47). Finally, individuals displaying the poor metabolizer phenotype appear less likely than extensive metabolizers to develop certain cancers (48). Although these findings have been confirmed (49–51) and are the source of extensive interest, the mechanism is unknown.

P-450$_{PA}$

The high-affinity phase of phenacetin O-deethylation is catalyzed by an enzyme, P-450$_{PA}$, which has been purified in this laboratory (32). Although this protein has still not been as extensively characterized as would be desired, a number of indirect lines of evidence indicate that this enzyme is the ortholog of rat P-450$_{ISF-G}$, a high-spin enzyme also termed P-450d and P-450H (3, 5).

Human P-450$_{PA}$ has been isolated in inactive form ("P-450d") by immunoadsorption methods and the N-terminal amino acid sequence has been reported (52); blotting and immunoinhibition studies with antirat P-450 preparations indicate that P-450$_{PA}$ is more similar to rat P-450$_{ISF-G}$ than P-450$_{\beta NF-B}$ (53). Other orthologs include mouse P_3-450 and rabbit P-450$_{LM-4}$. cDNA and genomic sequences of this P450IA2 gene have been derived from probes developed from experimental animals and have been published (54, 55). The only related gene is P450IA1, which codes for human P_1-450 (see below) (chromosome 15q22-qter—near MPI) (56).

To some extent, phenacetin O-deethylation cosegregates with debrisoquine 4-hydroxylation in humans (44), but the activities are clearly associated with different gene products (32). Although initial studies indicated that the phenacetin O-deethylase activity of different liver samples was not correlated with the amount of P-450$_{PA}$ detected by an anti-human P-450$_{PA}$ preparation, more recent results of studies where such analysis was done with heterologous anti-rat P-450$_{ISF-G}$ preparations indicate a good correlation (56a, 57). Further, levels of hepatic P-450$_{PA}$ and phenacetin O-deethylase activity are induced by cigarette smoking in humans (A. Boobis, personal communication) and it has been known for many years that in vivo phenacetin clearance can also be increased in smokers (58–60). Thus P-450$_{PA}$ appears to be essentially only a hepatic enzyme (A. Boobis, personal communication).

Other reactions catalyzed by the enzyme appear to be the N-hydroxylation of the pro-carcinogen 4-aminobiphenyl (56a, 57) and possibly other aromatic amines. Because of such bioactivation reactions carried out by the rat ortholog of this enzyme and its inducibility in humans by smoking, there is considerable interest in this enzyme in the area of chemical carcinogenesis (15).

P-450$_{NF}$

A series of immunochemically related preparations of human liver cytochrome P-450 were isolated in the author's laboratory and partially characterized (61). Subsequently, a human P-450 was isolated by Watkins et al (62) on the basis of immunochemical cross-reactivity with a rat liver P-450 induced by pregnenolone-16α-carbonitrile, and in the author's laboratory a P-450 was isolated on the basis of its ability to catalyze the oxidation of the calcium channel blocker nifedipine and termed P-450$_{NF}$ (63). These two latter protein preparations have been reported to have identical N-terminal sequences (62, 63a). In 1979 Kitada & Kamataki first reported the partial purification of a P-450 from human fetal liver (64). Subsequent work has shown that this protein, P-450 HLFa, and another, P-450 human-1 (65) are highly related to P-450$_{NF}$ (and P450p) (66–68), however, the N-terminal sequence of P-450HLFa has been reported to differ from the P-450$_{NF}$ and P-450p proteins (see above) and cDNA clones in two positions (67).

At this time, understanding the multiplicity of P-450$_{NF}$ and any related proteins that may exist is difficult. Genomic analysis suggests that three or more gene-like sequences may be present (69), and that several protein products may possibly be formed. One problem is that the proteins appear to lose catalytic activity upon purification; this is also a characteristic of the orthologous rabbit (P-450 3c) and rat P-450$_{PCN-E}$-related gene products in this P450IIIA gene subfamily (human chromosome 7 q 21.3–q 31.3) (69a). Several cDNA clones have been reported. Molowa et al (70) used overlapping partial cDNA clones to derive a sequence in which the N-terminal amino acid sequence of the protein matched that predicted by the 5' portion of the cDNA sequence. Two full-length cDNA clones have been sequenced in this laboratory (63a, 69). Since the clones of Molowa et al (70) were derived from the same cDNA library (prepared from a single liver) they may be compared to those characterized in this laboratory. The Molowa et al (70) "HLp" clone (constructed from overlapping partial clones) contains a 3-base insert compared to NF 25 (69) and also has a 20-base gap in the 3' non-coding region; about 41 base substitutions occur and 8 result in amino acid substitutions. The NF 10 clone (63a) is nearly identical to NF 25; it has a three-base deletion in the coding region, one base change in the 3'-non-coding region, and an 814-base 3' extension, with a second polyadenylation signal compared to NF 25. mRNA studies with diagnostic cDNA probes have revealed that, of the above three sequences, NF 25 is clearly predominant in all 12 human livers examined and NF 10 and HLp sequences are present at $\leq$ 10% the level of clone NF 25-specific mRNA (63a). However, the use of the extended 3' region (second polyadenylation signal of clone NF10) appears to be the basis of the small amount of higher molecular weight mRNA (3.0 kb) found. Although a relationship between mRNA and protein levels was not seen in limited preliminary studies (69), the level of the 2.2 kb mRNA species now appears to be well-correlated with the amount of P-450$_{NF}$-related protein and catalytically active protein (63a). Recently Komori et al (71) have isolated a partial-length cDNA clone from a human fetal liver cDNA library that is about 5% different in its sequence from the other clones (NF 10, NF 25, and HLp). Also, Gonzalez et al (39) have expressed a cDNA differing from NF 25 in only two bases (one amino acid) in COS kidney cells and have found catalytic activity towards nifedipine in a qualitative assay.

In vivo studies suggest the enzyme is inducible by compounds such as barbiturates, dexamethasone, and other steroids, and macrolide antibiotics, such as erythromycin (62, 72, 73). Further, individuals to whom such compounds have been administered tend to have higher levels of immunochemically-detectable P-450$_{NF}$ (or related proteins). In contrast to P-450$_{MP}$ (see below), P-450$_{NF}$ is localized in the centrilobular region of the liver (74, 75). The enzyme is localized in liver, but lower levels are also found in adult intestine (75, 76) and fetal kidney, adrenal, and lung (66, 77).

A number of compounds appear to be substrates for P-450$_{NF}$, as ascertained largely from immunoinhibition studies. These include nifedipine and more than 20 other 1,4-dihydropyridines (63, 78, 79), quinidine (N-oxygenation and 3-hydroxylation) (37), testosterone, androstenedione, progesterone, and cortisol (6β hydroxylation) (63, 80, 80a), 17β-estradiol (63) (2- and 4-hydroxylation), 17α-ethynylestradiol (2-hydroxylation) (81), dehydroepiandrosterone 3-sulfate (16α-hydroxylation) (67), erythromycin N-demethylation (62), and cyclosporin hydroxylation (82). Benzphetamine N-demethylation and aldrin epoxidation are catalyzed by the enzyme, but other P-450s also contribute (63). The catalytic activity of P-450$_{NF}$ is stimulated by human cytochrome b_5, and microsomal activity towards the substrates nifedipine and testosterone is inhibited by antihuman cytochrome b_5 (63). P-450$_{NF}$ is characteristically inactivated by complexation of a product of troleandomycin oxidation with the heme (62). In addition, mechanism-based inactivation is observed during the oxidation of 17α-ethynylestradiol, presumably due to heme N-alkylation (81).

The assignment of catalytic activities requires caveats in that the purified P-450$_{NF}$-related enzymes are relatively inactive and most of the assignments have been made on the basis of immunoinhibition studies. However, it is distinctly possible that closely related proteins may exist that the antibodies may not distinguish. Further work will be required before the unambiguous association of catalytic activities with discrete protein sequences can be made, although the report of Gonzalez et al (39) would strongly suggest that sequences of the cDNA clones PCN1 (39) and NF 25 (69) probably represent active nifedipine oxidase proteins.

Catalytic activities related to P-450$_{NF}$ and the levels of the protein and mRNA vary widely among individual humans (62, 63, 63a). The original report of Kleinbloesem et al (83) suggested that genetic polymorphism influenced levels of in vivo nifedipine oxidase activity. However, more recent findings with an expanded population suggest that the variation is unimodal as opposed to bimodal (84), which is consistent with the variation in mRNA levels as the basis of differences in levels of P-450$_{NF}$ and catalytic activity (63a, 70). It is of interest that P-450$_{NF}$ catalyzes the dehydrogenation of nifedipine and other 1,4-dihydropyridines (78); subsequent cytochrome P-450-catalyzed reactions include ester cleavage (85, 86; T. Funaki, F. P. Guengerich, P. Soons, & D. D. Breimer, unpublished) and methyl hydroxylation (86), so that the first metabolite must be measured in order to accurately monitor the variation among individuals.

P-450$_{MP}$

At least three proteins are found in this multigene family. The story goes back to some of the first human liver P-450 preparations purified to homogeneity (87), which subsequent studies have indicated are probably in this group (61,

88, 89). Using S-mephenytoin 4'-hydroxylase activity as a guide, Shimada et al (89) purified two forms of P-450, P-450_{MP-1} and P-450_{MP-2}. P-450_{MP-1} and P-450_{MP-2} had similar catalytic activities toward mephenytoin and other substrates (both K_m and V_{max}), N-terminal amino acid sequences, and spectral properties, and could not be distinguished using polyclonal antibodies. The two proteins could be distinguished by their apparent monomeric molecular weights and in vitro translation experiments indicated that they were products of different mRNAs (89); more recently monoclonal antibodies have been developed that recognize P-450_{MP-1} but not P-450_{MP-2} (23). Another protein, termed P-450_{MP-3} in this laboratory (23), has apparently also been isolated by other groups (90, 91). This protein has the same apparent monomeric molecular weight as P-450_{MP-1} in the author's laboratory, but monoclonal antibodies distinguish the two proteins (23). Lasker et al (91) reported the chromatographic and electrophoretic separation of proteins corresponding to P-450_{MP-1} and P-450_{MP-3} (some N-terminal amino acids differ from the reported amino acid sequences and those predicted by cDNA clones, however). P-450_{MP-3} apparently does not catalyze S-mephenytoin hydroxylation (23); some catalytic activity for benzphetamine and aminopyrine N-demethylation has been reported (91). Other proteins resembling P-450_{MP-1} have now been isolated (65, 91, 92). Work in this laboratory suggests that P-450_{MP-1} and P-450_{MP-2} catalyze the 4'-hydroxylation of S-mephenytoin and S-nirvanol (89) and the 3'-hydroxylation of hexobarbital (93). The oxidation of tolbutamide, a hypoglycemic agent, has reportedly been expressed in a trimodal genetic polymorphism (94); in vitro experiments suggest that P-450_{MP-1} catalyzes tolbutamide methyl hydroxylation (95), although selective inhibition by sulfaphenazole (P. K. Srivastava, F. P. Guengerich, unpublished results) and in vivo studies (95) raise the possibility that a related enzyme may be involved (P-450_{MP-3} is not active). The possible oxidation of related hydantoin and barbituate derivatives is considered elsewhere (89, 96). Lasker et al (91) report that purified P-450-C (P-450_{MP-1}?) catalyzed N,N-dimethylnitrosamine N-demethylation at a rate higher than P-450j (see below) in the presence of cytochrome b_5. Although anti-P-450j blocks ~75% of N,N-dimethylnitrosamine N-demethylation and the catalytic activity is well-correlated with immunochemically determined P-450j in a series of microsomal samples (97, 98), P-450_{MP-1} may also contribute to this activity.

As mentioned above, Lasker et al (91) reported enhancement of P-450_{MP-1} (P-450-C) activity towards N,N-dimethylnitrosamine by rat cytochrome b_5. Shimada et al (89) also reported that S-mephenytoin 4'-hydroxylation by P-450_{MP-1} and P-450_{MP-2} is stimulated by rat or human cytochrome b_5 and anti-human cytochrome b_5 inhibits human liver microsomal S-mephenytoin 4-hydroxylation.

Beaune et al (99) reported that human liver microsomal S-hydroxylation of

tienilic acid is inhibited by anti-P-450$_{MP-1}$ (termed P-450-8, see 61, 89). The antibody also inhibited in vitro covalent binding of tienilic acid to microsomal protein catalyzed by P-450. Also of interest is the observation that antibodies are found in sera of patients with cryptogenic chronic hepatitis and immunoallergic drug-induced hepatitis, including those cases induced by tienilic acid, and that these antibodies specifically recognize P-450$_{MP-1}$ (and P-450$_{MP-3}$) (99). The relationship of the auto-antibodies to the hepatitis that results is unknown. As in the case of P-450$_{NF}$, the existence of closely related gene products (P450IIC8/9, chromosome 10p) makes definitive assignment of catalytic specificity difficult and vector-based expression systems should prove useful (see below).

Genomic analysis suggests that ≥7 gene-like sequences are found in this human P-450 subfamily (23). Several cDNAs have been characterized. cDNA clone MP-8 was isolated by using anti-P-450$_{MP-1}$ (22). Of the animal P-450s, the most similarity is seen with sequence of rat P-450$_{UT-A}$ and P-450$_{UT-I}$ and rabbit P-450$_1$. Recently a nearly identical clone, containing only a six-base deletion in the coding sequence, has been isolated from another cDNA library by Komori et al (71). The isolation of a second cDNA clone, MP-4, from the same cDNA library as MP-8, shows only two (non-silent) base differences in the protein coding region from the MP-8 clone, but differs dramatically in the 3' nontranslated region (23); what appears to be essentially the same clone (2 base differences) has been isolated from another library by Yasumori et al (100). The N-terminal sequences predicted by the MP-4 and MP-8 cDNA clones match those determined by amino acid sequencing of both the P-450$_{MP-1}$ and P-450$_{MP-2}$ proteins (89). Although in vitro translation experiments clearly indicate that P-450$_{MP-1}$ and P-450$_{MP-2}$ proteins are coded for by different mRNAs (89), current evidence does not permit elucidation of the assignment of specific cDNAs for the two proteins. Recently both the MP-4 (101) and MP-8 (101a) cDNA sequences have been expressed in vector-based yeast systems. The MP-4-derived protein showed low catalytic activity for testosterone (6β) hydroxylation, 7-ethoxycoumarin O-deethylation, and benzo(a)pyrene hydroxylation (101). The MP-8-derived protein catalyzed the methyl hydroxylation of tolbutamide but not S-mephenytoin 4'-hydroxylation.

cDNA clones with predicted N-terminal amino acid sequences matching the P-450$_{MP-3}$ protein (23, 90, 91) have been isolated (23, 102, 103). Okino et al (102) also report an unusual mRNA splicing defect in one cDNA clone. The two clones isolated in this laboratory (MP-12, MP-20) are very similar to each other and differ in only 4 bases (and 4 amino acids) in the coding region. The MP-4, MP-8, and MP-12/20 clones can all be isolated from a single liver cDNA library and immunochemical and immunoelectrophoresis suggest that P-450$_{MP-1}$, P-450$_{MP-2}$, and P-450$_{MP-3}$ proteins can all exist together in a single liver (23); therefore, at least three genes in this P-450 family are

expressed at the protein level. Studies with specific oligonucleotide probes also indicate that mRNAs related to all three clones (MP-4, MP-8, MP-12/20) are found in a single liver (23). Fetal liver is devoid of P–450_{MP}-related proteins, mRNA, and catalytic activities (22, 89, 95). Immunohistochemical localization studies show a diffuse localization of protein (74), but the interpretation is obscured by the multiplicity. Further studies will be required to precisely define relationships of genes, proteins, and catalytic activities in this P-450 family.

The basis of the genetic polymorphism remains unclear. While fewer than 5% of American or Swiss Caucasians are of the poor metabolizer phenotype, the incidence rises to more than 20% in Japanese (104). Attempts to correlate in vitro levels of S-mephenytoin 4'-hydroxylase activity with levels of P-450_{MP-1} or P-450_{MP-2} or mRNAs related to either the MP-4, MP-8, or MP-12/20 cDNA clones have been negative (23). Meier & Meyer (105) have also reported that extensive and poor metabolizers of S-mephenytoin have similar amounts of protein detected by anti-P-450_{MP-1} and that the proteins isolated from the two groups of individuals using immunoelectrophoresis are indistinguishable by a number of criteria, including N-terminal amino acid sequencing (first 6 residues, not quantified). These observations support the view that a structurally defective protein, and not the amount of a specific protein, underlies the mephenytoin polymorphism. Meier et al (106) have also reported that liver microsomes of poor metabolizers phenotyped in vivo also show decreased in vivo S-mephenytoin 4'-hydroxylation. Both a decreased V_{max} and increased K_m were seen in the poor metabolizers. Interestingly, although S-mephenytoin 4-hydroxylation is subject to genetic regulation, the activity also appears to be inducible by rifampicin (107). The relationship between the genetically-influenced and inducible activities remains to be discerned. All proteins (and mRNA) related to the P-450_{MP} enzymes are absent in human fetal tissue (22, 89, 95, 108). Levels of both P-450_{MP} and P-450_{NF} are decreased in primary liver tumors (109).

P-450j

Wrighton et al (97, 98) used antibodies prepared to a rat liver P-450 termed P-450j to isolate an orthologous human liver P-450, also termed P-450j. The first work used immunoaffinity methods to isolate an inactive enzyme (97) but subsequently a catalytically active enzyme was isolated using chromatography (98). Lasker et al (91) have also isolated a similar enzyme, P-450 ALC. The purified enzyme resembles its rat liver homolog in catalyzing (low K_m) N,N-dimethylnitrosamine N-demethylation and cytochrome b_5 stimulates the reaction, primarily by lowering the K_m for the substrate (98). Aniline (91, 98), ethanol (91), and acetaminophen (110) are also oxidized by the enzyme. Song et al (111) have isolated both human cDNA and genomic clones; only a

single gene appears to be present (P450IIE1). The gene is about 10 kb long and has been sequenced (112); the coding sequences of the rat and human genes share 75% identity (111).

The rat gene is regulated in a complex pattern with hypomethylation accompanying increased expression during development—induction with ethanol, acetone, isoniazid, and pyrazole is not accompanied by increased mRNA levels (111, 112). In humans there are suggestions that alcoholics have increased levels of P-450j (97) although further studies will be required to validate this hypothesis and, if true, establish the mechanism and its relevance.

P-450-AA

Laniado-Schwartzman et al (113) recently reported the isolation of a human P-450 on the basis of its catalytic activity in the epoxidation of arachidonic acid. The protein appears to be distinct from any others as judged by the reported N-terminal amino acid sequence. The purified protein catalyzes the oxidation of arachidonic acid to a mixture of all four epoxides with a turnover number 37–fold higher than found in microsomes (expressed on the basis of total P-450). The purified enzyme also showed a relatively high rate of 7-ethoxyresorufin O-deethylase activity, which is somewhat surprising in light of the report of Shimada (114) that 7-ethoxyresorufin O-deethylase activity is well-correlated (in human liver microsomal samples) to levels of what appears to be $P\text{-}450_{PA}$, a distinct enzyme. Also, Pelkonen et al (115) report that cigarette smoking induces 7-ethoxyresorufin O-deethylation in human liver and that the activity is inhibited by monoclonal antibody MAb 1-7-1, which is known to recognize P_1-450. Further information concerning the structural and catalytic properties of this P-450 is not yet available.

P-450$_9$

Beaune et al (116) reported the purification of a novel form of human liver P-450, termed $P\text{-}450_9$. Immunochemical studies indicated that the enzyme is unrelated to human $P\text{-}450_{NF}$ or $P\text{-}450_{MP}$ or any rat liver P-450s examined. The level of this P-450 varied about four-fold among the nine individual liver samples examined and in some microsomal samples a second, higher M_r protein was also seen. The purified enzyme showed low rates of catalysis of 4-nitroanisole O-demethylation, benzo(a)pyrene hydroxylation, and 7-ethoxycoumarin O-deethylation.

P-450$_{10}$

Recently Beaune and his associates have isolated another form of human liver P-450, termed $P\text{-}450_{10}$ (P. H. Beaune, personal communication). The purified enzyme appears to be distinct from $P\text{-}450_{NF}$, $P\text{-}450_{MP}$, and $P\text{-}450_9$, but

catalytic and structural comparisons with other preparations are not yet possible.

pHP450(1)

Phillips et al (117) used a cDNA probe related to a phenobarbital-inducible rat P-450 (P-450$_{PB\text{-}D}$ variant) to screen a human liver cDNA expression library and isolated a partial-length clone (1.25 kb), the sequence of which was 51% identical to that of P-450$_{PB\text{-}D}$. However, more extensive sequence identity was found with the rat P-450a (P-450$_{UT\text{-}F}$) sequence (118). The human protein has not been isolated nor has the cloned sequence been expressed—the rat liver protein has very characteristic testosterone 7α-hydroxylase activity (119). It should be pointed out that human liver has relatively little testosterone 7α-hydroxylase activity (80), and in other instances apparently orthologous proteins can have quite different functions. The pHP450(1) cDNA probe hybridized to 2 mRNA bands (1850, 2750 bases) and to phenobarbital-inducible marmoset mRNA (118). Genomic blotting suggests that pHP450(1) is the product of a multigene family, tentatively assigned P450IIA and mapped to chromosome 19q13.1–13.2 (117, 118).

P_1–450

Although P_1-450 has never been isolated from human tissue, considerable information is available regarding the enzyme and its regulation. Studies in mice and rats indicated that the enzyme is associated with benzo(a)pyrene hydroxylase activity (120), and Jaiswal et al (121) used a mouse P_1-450 cDNA probe to screen a human MCF-7 breast carcinoma cell cDNA library and isolate a human P_1-450 cDNA clone, which shares greater than 80% sequence identity with the mouse and rat sequences (P450IA1, chromosome 15q22-qter, near MPI). Genomic clones have also been isolated and sequenced (56, 122, 123). The human genomic sequence appears similar to that of the mouse and conclusions about regulation can probably be extended to the human gene. Sequence similarity is shared only with P-450$_{PA}$ (72%).

In experimental animals orthologs of P_1-450 are induced in both the liver and extrahepatic tissues by polycyclic hydrocarbons and other compounds (124). Several lines of evidence suggest that, in humans, P_1-450 is primarily an extrahepatic enzyme. While P-450$_{PA}$ and its associated activities are induced by cigarette smoking and consumption of charbroiled meats (45), women who smoke cigarettes show greatly elevated levels of benzo(a)pyrene hydroxylase in placentae, but smokers do not show elevated hepatic enzyme activity (115). Further, immunochemical screening of human liver microsomes suggests that P-450$_{PA}$ is often present but that proteins more closely related to P_1-450 occur rather infrequently (52, 53). Although such results could be misconstrued because of differences in apparent monomeric molecu-

lar weights of orthologous proteins, human liver mRNA analysis with cDNA probes that distinguish between P-450$_{PA}$- and P$_1$-450-related sequences suggests that P$_1$-450 levels are very low in most human livers (125). Thus, although the possibility exists that exposure of humans to high levels of chlorinated hydrocarbons (polyhalogenated biphenyls or dioxins) or other inducers may induce hepatic P$_1$-450 in humans, P$_1$-450 should probably be considered primarily an extrahepatic enzyme in humans. P-450$_{PA}$ is not expressed appreciably in extrahepatic tissues. Studies with peripheral blood cells indicate that levels of both basal and hydrocarbon-inducible benzo(a)pyrene hydroxylase activity vary considerably among individuals (126); levels of P$_1$-450 mRNA are well correlated with levels of catalytic activity (56). Exactly how the gene is regulated in humans is not known.

Many polycyclic hydrocarbons appear to be inducers of and substrates for human P$_1$-450. While regulation of the 5' flanking sequence has been studied with use of chimeric constructs (54, 122), extensive studies of catalytic activity of the protein itself have not been carried out and the enzyme has not been purified. While some inferences can be drawn from studies with orthologous animal proteins, some differences may be expected since mouse P$_1$-450 and rat P-450$_{\beta NF\text{-}B}$ differ remarkably in their warfarin hydroxylase activities (21).

P450arom

The aromatase reaction is an overall 6-electron oxidation in which androgens such as testosterone are converted to estrogens. The reaction serves as a key control point in hormone balance and is also the source of considerable interest in cancer therapy because of the promotional nature of estrogens (127). The enzyme is a microsomal P-450; it is localized primarily in steroidogenic tissues such as ovary and in the placenta and breast. Mendelson et al (128) partially purified human placental P-450 aromatase and developed a monoclonal antibody; P-450 purified by the use of immunoaffinity chromatography had very low catalytic activity. Chen et al (129) partially purified human placental aromatase and reported an N-terminal amino acid sequence. The purification of P-450arom from pooled human placenta has been reported by Tan & Muto (130) and by Kellis & Vickery (131). The purified enzyme is catalytically active, utilizing androstenedione, testosterone, or 16α-hydroxytestosterone as substrate; the K_m for androstenedione is 0.06 μM (131). Presumably the substrate specificity of this enzyme will be quite limited, in contrast to the hepatic enzymes that oxidize xenobiotics. Vickery and his associates (132) have used a series of ligands in spectroscopic studies to map the distance and orientation between the substrate binding site and the heme iron.

Evans et al (133) reported the isolation of a cDNA clone from a human

placental library (1.8 kb) that recognizes ≥ 3 mRNA sizes (2.4, 2.7, and 3.0 kb). Recently Chen et al (134) have sequenced a genomic clone.

P-450$_{C\text{-}21}$

P-450$_{C\text{-}21}$ is localized in the zona fasciculata of the adrenal cortex and is not expressed in liver. The enzyme hydroxylates 17-hydroxyprogesterone at the 21-position to form 11-deoxycortisol. When the enzyme is genetically impaired, congenital adrenal hyperplasia results. Cortisol levels are low, ACTH levels are high, and androgens are elevated. In its severe form, this is termed "salt wasting" syndrome and has a number of deleterious symptoms. The disease affects about $1/10^4$ children born and is considered a major inborn error of metabolism.

Although the human enzyme has never been purified, much is known about the genes and interest is high because of the severity of the genetic disease. Two genes are located on chromosome 6 near the complement C4 gene (135). White et al (136, 137) used bovine P-450$_{C\text{-}21}$ cDNA probes and showed the existence of two genes in tandem: "A" (P450XXIA1) is a pseudogene and "B" (P450XXIA2) is expressed. Both genes are about 3.4 kb in length, with 10 exons; gene A, when compared to B, has a 1-base insertion, an 8-base deletion, and a transition in the coding region that result in premature termination (138–140).

Homozygous recessive individuals were missing one of two *Taq*I or *Eco*RI bands upon genomic analysis (136). In some cases of congenital adrenal hyperplasia, the complement C4B (histocompatibility) gene also appears to be deleted along with the P-450$_{C\text{-}21}$ B gene (141). In some of the patients examined, the B gene is converted to the A by recombination and results in gene conversion (139, 142, 143). Analysis of a group of severely afflicted patients indicated that point mutations of gene B were detected in about one half, complete deletion is about one third, and gene B to A conversion in about one tenth of the individuals (142). Rccently Matteson et al (143) have concluded that the reported gene B deletions are actually gene conversions, unequal crossovers, or polymorphisms.

P-450$_{17\alpha}$

P-450$_{17\alpha}$ is expressed in human adrenal cortex, testis, and ovarian theca cells, but not in liver or other tissues. The protein is a single P-450 catalyzing both 17α-hydroxylase and 17,20-lyase activities in the biosynthesis of steroid hormones from pregnenolone. The protein has not been purified from human tissue but cDNA and genomic clones have been isolated and sequenced (144). The single gene (6.6 kb) is placed in the P450XVIIA1 family (chromosome 10). The intron/exon boundaries and protein hydropathy plots resemble those of P-450$_{C\text{-}21}$ (P450XXI) although only 29% sequence identity is observed in

the coding regions. In the human adrenal P450 $_{17\alpha}$ expression is under ACTH regulation via cAMP, while in the testes expression is under gonadotropin control via cAMP. An autosomal recessive deficiency of human 17,20-lyase activity exists in which 17α-hydroxylase activity remains normal (145) although several lines of evidence indicate that only one protein and gene are associated with both catalytic activities (146–148). Recently Kagimoto et al (149) have sequenced the exons of the gene from an individual deficient in both 17α and 17,20-lyase activities and identified a 4-base duplication in exon 8 that yields an altered protein (C-terminal region). Another individual with combined deficiency was found to have a (TGA) stop codon at amino acid 17 of exon 1 (149a).

SUMMARY AND FUTURE CONSIDERATIONS

At this time it appears that at least 16 microsomal P-450 genes are expressed in humans. Of these, four appear to be primarily extrahepatic. Nearly all of the hepatic enzymes characterized to date have been described in terms of their catalytic activities, if they exist, for drugs and other xenobiotics. In some cases, both proteins and genes have been characterized. However, in other cases the multiplicity in gene families requires that further characterization of genes and proteins and enzyme expression will be needed before a full assignment of proteins to genes can be made. In every case, much remains to be learned about structure-function relationships.

What then, does the future hold in the study of human P-450 enzymes?

First, more human P-450s undoubtedly exist and remain to be isolated and characterized. The P-450s that have been studied need to be further characterized. In many cases, assignment of proteins to discrete DNA sequences needs to be made, and much remains to be learned about gene structure and regulation. Site-directed mutagenesis and expression in vector-based systems will be necessary to probe details of binding of individual substrates. The elucidation of three-dimensional structures of the human P-450s would be of tremendous benefit in this regard and will ultimately have to be achieved. This is a formidable task, since the large size of the P-450s and their tendency to aggregate renders NMR approaches difficult. There are potentially useful strategies for crystallizing intrinsic membrane-bound proteins and possibly deriving functional P-450s without membrane-binding domains (150).

Much insight into human P-450s has been possible because of the polymorphisms that were first described in vitro. In the future it would be useful to identify new and distinct polymorphisms. These may be observed in clinical and other in vivo studies, and in the future differences first identified at the restriction fragment length polymorphism level may lead to useful insights regarding P-450 structure and function.

Questions arise as to the in vivo relevance of variations in P-450. There are now many relatively specific inhibitors of individual P-450s that can be utilized in vitro and in vivo. For instance, quinidine is a strong inhibitor of $P\text{-}450_{DB}$ (36) and sulfaphenazole inhibits tolbutamide hydroxylation (see above). Under the appropriate conditions, these compounds can be given to people to examine the effects of attenuating one P-450.

In the future we should also be able to utilize in vitro experiments to predict in vivo results. For instance, the specificity of individual forms of P-450 toward a new compound under consideration may be examined by using enzyme reconstitution, immunoinhibition, and expression systems. Such experimcnts may be of particular value in determining which P-450s activate and detoxicate individual carcinogens. In the case of drugs, it is possible to verify the hypothesis made with in vitro data in in vivo experiments. In this regard there are physiologically-based pharmacokinetic models in which knowledge of the kinetic parameters of rate-limiting steps can be utilized in predicting in vivo metabolic differences among species (151, 152). Although these models may find the most application in studies with pharmaceuticals, there are also some situations in which pro-carcinogens and -toxicants may also be considered. For instance, when sensitivity permits, studies can be done in workers when exposure to industrial compounds does not exceed the maximum allowed levels. Accidental high level exposure situations may also provide information that can be used.

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