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REGIOCHEMISTRY OF CYTOCHROME P450 ISOZYMES

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INTRODUCTION

The ability of animals to metabolize xenobiotics has been acquired over a period of more than 2 thousand million years during the process of adaptation to the environment. The acquired character has been accumulated and conserved in the genome as genetic information. Thus, the ability of animals to metabolize the large number of chemicals produced by modern chemical industries must be an inheritance of enforced contact with natural xenobiotics. In early studies with liver microsomes, the substrate specificity of cytochrome P450 (P450) was thought to be very general, but investigators now believe that its broad specificity can be explained in terms of an integrated system with numerous P450 isoenzymes. Regio- and stereoselective aspects of drug metabolism by P450 are important concerns in studies of the pharmacokinetics of racemates or in the extrapolation of metabolic profiles of drugs from laboratory animals to humans. In this review, we summarize current knowledge concerning the regioselectivity and stereoselectivity of the major P450 isozymes.

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PREDICTION OF REGIOSELECTIVITY OF P450 BY CALCULATION CHEMISTRY

Stereochemical aspects of drug metabolism are the subject of renewed interest and the object of many research efforts. In the protein moiety of P450, the conformational changes caused by substrate binding are thought to be important for enzyme function; the selective nature of this substrate binding of P450s may be the primary determinant of stereo- and regiospecificity. The reactivity of the substrate to active oxygen generated by P450 is another determinant of regioselective metabolism and has been examined from a theoretical perspective. Results from experiments with liver microsomes from differently pretreated rats have demonstrated that the observed regioselectivity for the aromatic hydroxylation of monofluoroanilines is not predominantly determined by the active site of the cytochrome P450s (1). To investigate the underlying reason for the observed regioselectivity, semiempirical molecular orbital calculations were performed and showed that neither the frontier orbital densities of the LUMO/LUMO+1 (the lowest unoccupied molecular orbital) of the monofluoroanilines nor the spin densities in their $NH^{\bullet}$ radicals could account for the observed regioselectivity. The frontier orbital densities of the HOMO/HOMO-1 (the highest occupied molecular orbital and the secondary highest orbital) of the monofluoroanilines, however, qualitatively correlate with the regioselectivity of the aromatic hydroxylation. The results suggest that P450-catalyzed aromatic hydroxylation of monofluoroanilines proceeds by an electrophilic attack of the $(FeO)^{3+}$ species of P450 on a specific carbon atom of the aromatic ring. Subsequently, the regioselectivity of hydroxylation of five fluorobenzene derivatives was studied using ^{19}F NMR for identification of the various phenols in the urine of rats exposed to these benzenes (2). The regioselectivities observed for the aromatic hydroxylation of the fluorobenzenes in these in vivo experiments were shown to correlate with the values predicted for regioselectivity, within 6% accuracy, on the basis of HOMO/HOMO-1 frontier orbital density.

Recently we have also found that the occupied frontier orbital densities predict the site of oxidation at aliphatic C–H bonds in xenobiotics by P450 (3, 4). The semiempirical quantum chemical method, PM3, was used to develop a predictive model for P450 hydrogen abstraction reactions. The preferential oxidation at carbon atoms adjacent to nitrogen and oxygen atoms and benzylic or allylic carbon atoms was accounted for by the calculated orbital densities on the C–H bonds. A chemical computational method, AM1, was used to obtain an estimate of the ionization potential in radical formation produced by hydrogen atom abstraction (5). In summary, the relatively simple procedure of optimizing the geometry of any compound

and its potential radicals may be useful in predicting the relative potential for hydrogen atom abstraction at the various positions, neglecting binding and steric constraints imposed by the enzymes. Although a selective substrate binding of P450s may be the primary determinant of the stereo- and regiospecificity for the enzyme, the site on the substrate attacked by the active oxygen from P450 may be predicted from theoretical considerations.

SUBSTRATE SPECIFICITY AND REGIOSELECTIVITY OF P450

During the last two decades, major efforts have been directed at the isolation and characterization of P450 isozymes, and considerable information is now available on the substrate specificity of homogeneous P450 preparations. In addition, the recent development of molecular biology has allowed us to express P450 from cDNA and to examine relationships between structure and function. Since the substrate specificity of P450 has recently been reviewed by Guengerich (6), Distlerath & Guengerich (7), Schwab & Johnson (8), and Soucek & Gut (9), we address this point only briefly. P450 isozymes responsible for steroidogenesis (10) seem to have rigid substrate specificity, but their function in xenobiotic metabolism has not been fully assessed. Examples are $P450_{SCC}$ (CYP11A1) (11, 12), $P450_{11\beta}$ (CYP11B1) (13, 14), $P450_{aldo}$ (CYP11B2) (15), $P450_{arom}$ (CYP19) (16), $P450_{17\alpha}$ (CYP17) (17), and $P450_{21\alpha}$ (CYP21) (18, 19). In addition, $P450_{14DM}$ (CYP51) (20, 21) and $P450_{7\alpha}$ (CYP7) (22), which are involved in the synthesis of cholesterol and bile acids, may be included among the isozymes that have rigid selectivity.

The other examples with high substrate specificity are the CYP4A subfamily P450s, which selectively hydroxylate the ω-position of fatty acids and prostaglandins. Although several fatty acids with different chain lengths can be substrates of a single P450 enzyme belonging to the CYP4A subfamily (23), this isozyme group has minimal ability to metabolize compounds other than fatty acids (24–27). Bacterial P450, $P450_{cam}$, is also an example of a form that hydroxylates camphor specifically (28), although some low molecular weight compounds such as adamantanone (28) and ethylbenzene (29) are metabolized. The high specificity of $P450_{cam}$ for camphor is demonstrated by the fact that thiocamphor, camphane, and norcamphor, compounds closely related to camphor, are far less effective substrates of $P450_{cam}$ (30, 31).

Regioselective Hydroxylation of Steroid Hormones

The P450s belonging to CYP1, -2, and -3 families are multifunctional and show a wide spectrum of substrate specificity. Many of these isozymes catalyze the regioselective hydroxylation of testosterone, and this is one of

the useful markers for the respective P450 forms (6, 9, 32). However, in contrast to the P450s participating in steroidogenesis and fatty acid/prostaglandin metabolism, many P450s in the CYP1 to 3 families oxidize xenobiotics. Benzphetamine, aminopyrine, aniline, 7-ethoxycoumarin, *p*-nitroanisole, and benzo[*a*]pyrene are the substrates used most frequently. The hydroxylation of these substrates is almost isoform-specific, but the specificity is not definitive (6, 8, 9, 33). While limited numbers of P450s show very strong activities toward the six compounds above, a number of P450s possess minor activities. A more detailed description of the substrate specificities of P450s belonging to the CYP1, 2, and 3 families is found in other recent reviews (6–9).

The hydroxylation of testosterone is one of the best examples in which the isozyme-specific regioselectivity has been elucidated with reconstituted systems containing highly purified P450 preparations. Moreover, this information has been accumulated for many P450s isolated from various animal species including man. Results of these studies are summarized in Table 1. The regioselective hydroxylation of androstendione (36, 40, 57, 58, 67) and progesterone (36, 45, 52, 54, 60, 68, 69) by purified P450s has also been reported. Although the structures of testosterone and these two steroids differ in only the D-ring, the rate of hydroxylation catalyzed by a particular P450 isozyme is sometimes different for the three steroids. For example, the phenobarbital-inducible CYP2B1 and CYP2B4 hydroxylate the 16-position, but the rates are greatly different among the three compounds (36, 40, 57, 58). The same findings are obtained for CYP2C11 (36), CYP2C13 (52), and P450-D-1 (CYP2C) (60). The regioselectivity and stereoselectivity are also altered by the different steroids. That is, while testosterone is hydroxylated at the 6β-position by CYP2C13, this P450 hydroxylates progesterone at the 16α- as well as the 6β-position (69). CYP2A2-mediated hydroxylation is one of the examples in which different amounts of stereoisomers are produced, depending on the substrates used; the ratio of 15α/15β-hydroxy products is 13.2 for testosterone and 0.39 for progesterone (52). These examples indicate that the regioselectivity and stereoselectivity of a P450 enzyme in the hydroxylation of steroids varies with only minor changes in the structure of the D-ring. The metabolism of estradiol by purified P450s at the 2-, 4-, 6β-, 15α-, 16α-, and 17-positions has also been reported (70). The results indicate that estradiol is not hydroxylated at the corresponding sites of testosterone and progesterone; while CYP2A2 and CYP2B1 hydroxylate the 15α- and 16α-positions of testosterone, respectively, the same sites of estradiol are metabolized at minor rates by these P450s (70). A P450 isozyme that is highly active for estradiol 2- and 16α-hydroxylation but not for hydroxylation of testosterone, androstendione, and progesterone has been isolated (71). Thus, the aromaticity of the A-ring

Table 1 Regioselective hydroxylations of testosterone in the reconstituted P450 system

Position of hydroxylation	Isozyme	Species	Reference
2α-Hydroxylation	CYP2C11	Rat	34–38
6β-Hydroxylation	CYP1A1	Rat	39
	CYP1A2	Rat	39
	CYP1A2	Rabbit	40
	CYP2C13	Rat	34, 35, 37, 38
	CYP3A1	Rat	41, 42
	CYP3A2	Rat	42, 43
	CYP3A3/4	Human	44, 45
	CYP3A5	Human	46
	CYP3A7	Human	45
	CYP2C3	Rabbit	40, 47
	CYP3A6	Rabbit	40
	P450FA (CYP3A)	Monkey	48
7α-Hydroxylation	CYP2A1	Rat	36, 49, 50
	P450H (CYP1A)	Hamster	51
	P450L (CYP2A)	Hamster	51
15α-Hydroxylation	CYP2A2	Rat	37, 38, 52, 53
	Cyp2a4/5	Mouse	54
	CYP2C12	Rat	38, 55
	CYP2G1	Rabbit	56
16α-Hydroxylation	CYP2B1	Rat	49, 57, 58
	CYP2C11	Rat	34–38
	$P450_{CBD}$ (Cyp2b)	Mouse	59
	CYP2B4	Rabbit	40
	CYP2C3	Rabbit	40, 47
	CYP2G1	Rabbit	56
	P450-D-1 (CYP2C)	Dog	60
16β-Hydroxylation	CYP2B1	Rat	57, 58
	$P450_{CBD}$ (Cyp2b)	Mouse	59
	$P450_{THC}$ (Cyp2c)	Mouse	61
	$P450_{GP-1}$ (CYP2B)	Guinea pig	62
	P450CMLa (CYP2B)	Monkey	63
	P450-HM2 (CYP2C)	Human	45
17-Oxidation	CYP2B1	Rat	49, 57, 58
	CYP2C11	Rat	34, 35
	$P450_{CBD}$ (Cyp2b)	Mouse	60
	CYP2A10	Rabbit	56
	P450-MK-1 (CYP2C)	Monkey	64
19-Hydroxylation	CYP2G1	Rabbit	56
	P450arom (CYP19)	Human	16, 65, 66

structure as well as the structure of the D-ring affects extensively the regioselectivity in the steroid hydroxylation by P450.

Alteration in specificity of steroid hydroxylation is also seen for the different members of P450 belonging to the same subfamily. Among the rat CYP2C subfamily, only CYP2C11 is able to catalyze testosterone 2α-hydroxylation effectively; CYP2C6, 7, 12, and 13 have little or no activity (35, 37, 38, 55, 58), although these four P450s are highly homologous with each other. Presumably, the P450s belonging to the dog CYP2C subfamily lack the ability of testosterone 2α-hydroxylation, because in contrast to rats, dog liver microsomes lack this metabolic activity (60). A case of different stereoselectivity of hydroxylation mediated by P450s belonging to the same subfamily is exemplified by the following. Rat CYP2B1 (57, 58) and dog CYP2B11 (67) hydroxylate testosterone or androstendione at both the 16α- and 16β-position almost equally, whereas guinea pig and monkey isozymes give only the 16β-isomer (62, 63) and rabbit isozyme gives only the 16α-isomer (40).

Regioselective Metabolism of Xenobiotics

Numerous examples of the P450 isozyme-dependent metabolism of xenobiotics have been reported (6–9). However, many of these lack information on regioselectivity. For instance, although benzphetamine *N*-demethylation is widely used to monitor some types of P450s, conceivably a P450 isozyme would favor hydroxylation at a position different from the *N*-methyl group, even if this isozyme has minor *N*-demethylase activity. Benzphetamine is metabolized via routes other than *N*-demethylation (72, 73). Highly purified CYP2C11 hydroxylates the methyl (formaldehyde formation), benzyl (benzaldehyde formation), and methylene carbon (phenylacetone formation) positions of benzphetamine (1 mM) at a ratio of 180:7:1, whereas CYP2C3 gives these products with a ratio of 7:2:1 (Yamada et al, unpublished observation). In this case, the rate of methylene hydroxylation by CYP2C3 is over 20 times greater than that by CYP2C11 while the rates of methyl hydroxylation are almost comparable. In other words, in a comparison of CYP2C3 and 2C11, only the former is an effective catalyst for phenylacetone formation although both are equally effective in the *N*-demethylation. Thus, the substrate specificity of P450 should not be defined by measuring catalytic activity toward a single oxidation site on substrate.

However, as already described, regioselectivity in metabolite formation has been determined for only a limited number of xenobiotic substrates. The major reason for insufficient information is that attention has been focused on identifying the P450 isozymes. For this purpose, researchers have utilized a single reaction for one substrate such as benzphetamine *N*-demethylation without monitoring other metabolites. Another reason is

that, from a toxicological perspective, only metabolites causing toxic action have attracted the attention of researchers. If a substrate gives only one oxygenated metabolite, this should be recognized as a highly regioselective reaction. However, in many studies measuring a single product, it is not known whether the product is the sole metabolite or whether other products have not been analyzed. With these issues in mind, we list here examples of P450-mediated regioselective metabolism of a limited number of compounds that give multiple metabolites with reconstituted systems containing purified P450 from CYP1A, 2, 3A, and 4A families (Table 2). Except for fatty acids and related compounds, all listed substrates are foreign compounds.

Warfarin is one of the most extensively examined compounds, and information about enantio- and regiospecific hydroxylation has been accumulated using a number of purified P450s from rat, rabbit, and dog liver microsomes (Table 2) (33, 67, 82, 83). In addition, experiments with antibody inhibition and enzyme expression from cDNA have revealed enantio- and regioselectivity of mouse (134) and human (135, 136) P450s that is not available with the purified forms. The composition of P450 isozymes in human liver can be predicted by the analysis of metabolites formed from *S*- and *R*-warfarin (137, 138). Warfarin can be utilized as a probe for the estimation of UDP-glucuronyltransferase and sulfotransferase as well as P450 activities (139). Based on the highly regiospecific and stereospecific nature of warfarin hydroxylation, an attempt at photoaffinity labelling of P450 by azidowarfarin was made (140, 141).

A cautionary note is needed in determining the oxygenation site of the aromatic ring in phenol formation. If the precursor is an epoxide, different regioisomeric phenols can be generated. Alternatively, it is possible that one phenolic metabolite is produced from different epoxide precursors. Therefore, it is necessary to identify the epoxidation site of the substrate by analyzing hydrolyzed products or thiol adducts. Concerning this point, epoxy-metabolites of many polycyclic aromatic hydrocarbons listed in Table 2 have been analyzed as the dihydrodiols after hydrolysis with epoxide hydrolase supplemented in the incubation mixture. Although regioisomeric metabolites from a series of dichlorobiphenyls have been analyzed (142), we ignored these results in Table 2. The reason is that the initial position of oxygenation cannot be readily specified for these reactions because many of the reported monophenol metabolites have their hydroxyl groups at adjacent positions (i.e. 3-hydroxy and 4-hydroxy).

Table 2 shows only a few cases of metabolism mediated by the CYP2E subfamily P450. This is due to the fact that most of the excellent substrates of CYP2E P450 are simple compounds of low molecular weights such as alcohols and nitrosamines (6). Purified CYP2E P450 is reported to mediate

Table 2 Substrates giving multiple metabolites in the reconstituted system containing purified P450 of the CYP 1A, 2, 3A, and 4A family

Substrate	Reactions	Isozyme examined	References
n-Hexane	1-, 2-, and 3-Hydroxylation	CYP1A1, 2B1	75, 76
trans-1-Phenyl-1-butene	Formation of 1-phenyl-1- and 2-butanone	CYP2B1	77
Toluene	Benzyl-hydroxylation, *o*- and *p/m*-hydroxylation	CYP2B1, 2B2, 2C6	78, 79
Camphor	3-endo-, 5-exo-, and 5-endo-Hydroxylation	CYP2B4	80
Valproic acid	4- and 5-Hydroxylation, desaturation at 4–5 position	CYP2B	81
Warfarin	4-, 6-, 7-, 8-, and 10-Hydroxylations	Isozymes of CYP1A, 2A, 2B, 2C, 3A subfamily	33, 67, 82, 83
Scoparone	*O*-Demethylations at 6- and 7-methoxy groups	Isozymes of CYP1A, 2B, and from hen liver	84, 85
Nicotine	5′-Hydroxylation, *N*-methyl hydroxylation	CYP1A1, 1A2, 2B4, 2C3, 2E1, 3A6	86
Strychnine	2-, 16- and 22-Hydroxylations, 21,22-oxide formation, *N*-oxygenation	CYP2B1, 2B2	87
Senecionine	Desaturation of pyrrolizidine nucleus, *N*-oxygenation	CYP1A1, 2B1, 2C11	88
Theophylline	Demethylations at N_1- and N_7-, and N_3- and N_7-methyl group, 8-oxygenation	CYP1A1, 1A2, 2C3	89

Δ^9-Tetrahydrocannabinol	8α-, 8β, 11-, and 3′-Hydroxylation	CYP2A2, 2C8, 2C9, 2C11, 2C13, $P450_{THC}$ (CYP2C)	90–92
Aminopyrine	3-Methyl hydroxylation, *N*-demethylation	Isozymes of CYP1A, 2A, 2B, 2C, 2D subfamily	93
Antipyrine	3-Methyl and 4-hydroxylation	CYP1A1, 2B1, 2B2	94
Propranolol	4-, 5-, and 7-Hydroxylations, *N*-depropylation	Isozymes of CYP1A 2A, 2B, 2C, 2D, 2E, 3A subfamily	95, 96
Encainide	*N*- and *O*-Demethylation	$P450_{UT-H}$ (CYP2D)	96
Metropronol	α-Hydroxylation, *O*-demethylation	CYP2D6	97
Sparteine	Δ^2- and Δ^5-Oxidation	CYP2D6	97
Mephenytoin	*N*-Demethylation, 4′-hydroxylation	CYP2C9, 2C11, 3A1, 3A2	98–100
Lidocaine	*N*-Deethylation, methyl hydroxylation, aromatic (3-) hydroxylation	Isozymes of CYP1A, 2C, 2B, 3A subfamily	101, 102
Quinidine	*N*-Oxygenation, 3-hydroxylation	CYP3A3/4	103
Cyclosporine	*N*-Demethylation and other hydroxylations	CYP1A2, 2B4, 3A6	104
p-Tolylethyl sulfide	*S*-Oxygenation, methyl hydroxylation	CYP2A1, 2B1, 2B2, 2C6, 2C11, 2C12	36, 78, 79
Vinylidene chloride	Formation of dichloroacetaldehyde and chloroacetic acid	Isozymes of 1A, 2B, 2C, 3A subfamily	77
1-(2-Chloroethyl)-3-(cyclohexyl)-1-nitrosourea (CCNU)	2 (*cis* and *trans*)-Hydroxylation, 3 (*cis* and *trans*)-hydroxylation, 4 (*cis* and *trans*)-hydroxylation	CYP1A1, 2A1, 2B1	105
Aflatoxin B_1	Formation of aflatoxin Q_1, M_1, P_1	CYP2B1, P450mt3	106
Biphenyl	2- and 4-Hydroxylation	CYP1A1, 1A2	107
Benz[*a*]anthracene	5,6- and 8,9-Epoxidation	CYP1A1	108
Benz[*a*]anthracene 1,2-dihydrodiol	Bis-dihydrodiols and other metabolites (see reference for metabolic sites)	CYP1A1	109
Benz[*a*]anthracene 3,4-dihydrodiol	Bis-dihydrodiols and other metabolites (see reference for metabolic sites)	CYP1A1	110

Table 2 (*Continued*)

Substrate	Reactions	Isozyme examined	References
8-Methylbenz[*a*]anthracene	Dihydrodiols, phenols, and hydroxymethyl and formyl metabolites (see reference for metabolic sites)	CYP1A1, 2B1	111
7,12-Dimethylbenz[*a*]anthracene	Dihydrodiols, phenols and hydroxymethylmetabolites (see reference for metabolic sites)	CYP1A1, 1A2, 2B1, 2B2, 2C11, P450PCN (CYP3A)	112
Benzo[*c*]phenanthrene	3,4- and 5,6- Epoxidation	CYP1A1	113
Benzo[*c*]phenanthrene 3,4-dihydrodiol	Bis-dihydrodiols and other metabolites (see reference for metabolic sites)	CYP1A1	114
Cyclopenta[*cd*]pyrene	3,4- and 9,10-Epoxidation	CYP1A1, 1A2, 3A4	115
Benzo[*a*]pyrene	Dihydrodiols, qinones, and phenols (see reference for metabolic sites)	CYP1A1, 1A2, 2B1, 2B2, 2C11, P450PCN (CYP3A)	112, 116, 117
1-Nitropyrene	Oxides, dihydrodiols, phenols (see reference for metabolic sites)	CYP1A1, 2B4, 2C3, 3A6	118
1-Naphthylamine	*N*- and 2-Hydroxylation	Isozymes of CYP1A, 2B, 2C, 3A subfamily	119
2-Naphthylamine	*N*-, 1-, and 6-Hydroxylation	Isozymes of CYP1A, 2B, 2C, 3A subfamily	119
2-Aminofluorene	*N*, 5-, and 7-Hydroxylation	Isozymes of CYP1A, 2B, 2C, 3A subfamily	119
2-Acetylaminofluorene	*N*, 1-, 3-, 5-, 7-, and 9-Hydroxylation	Isozymes of CYP1A, 2A, 2B, 2C, 3A subfamily	120
4-Aminobiphenyl	*N*-, 3-, 2′-, and 4′-Hydroxylation	Isozymes of CYP 1A, 2B, 2C, 2D, 3A subfamily	121

4,4'-Methylene-bis(2-chloroaniline)	*N*-, benzyl-, and 6-Hydroxylation	Isozymes of CYP1A, 2B, 2C, 2D, 3A subfamily	121
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)	*N*- and 4'-Hydroxylation	Isozymes of CYP1A, 2B, 2C, 2E subfamily	122
Azoprocarbazine	Two different *N*-oxygenations at an azo group	Isozymes of CYP1A, 2B, 2C, 3A	123
Lauric acid, myristic acid, palmitic acid, stearic acid	ω- and ω-1-Hydroxylation	Isozymes of CYP1A, 2A, 2B, 2C, 2E, 4A subfamily	23, 27, 124–127
Arachidonic acid	Monohydroxylated metabolites, epoxides	Isozymes of CYP1A, 2A, 2B, 2C, 2E, 4A, and βNF_{AA}	126–132
Prostaglandin E_1, E_2	ω-, ω-1-, and ω-2-Hydroxylation	Isozymes of CYP2A, 2C, 2E, 4A	23, 27, 124, 133
Prostaglandin $F_{2\alpha}$, D_2	ω- and ω-1-Hydroxylation	CYP4A4	27
Prostaglandin A_1, A_2	ω- and ω-1-Hydroxylation	Isozymes of CYP2B, 2C, 4A	23, 27, 124, 126
Leukotriene B4	ω- and ω-1-Hydroxylation	CYP4A1	23

the metabolism of methylalkylnitrosamines to nitrite (143, 144). Although the detailed mechanism is unknown, it is proposed that nitrite is derived from nitric oxide, which is formed by P450-catalyzed reduction of nitrosamine (145). Dialkylnitrosamines, on the other hand, generate alkylaldehydes and a cation that is the ultimate genotoxic species. A portion of this alkyl cation is occasionally converted to alkene by the elimination of one proton (146). These metabolites of nitrosamines are believed to be formed from a common α-hydroxylated metabolite that is initially produced by P450. Another example of a common intermediate that gives multiple metabolites is parathione. Parathione is metabolized to paraoxone, diethylphosphorothionate, and diethylphosphate by highly purified P450s (147, 148), but these metabolites are thought to be formed nonenzymatically from a *S*-oxygenated intermediate that is produced initially (147).

ACTIVE SITE STRUCTURE OF P450 AS A DETERMINANT OF REGIOSELECTIVITY

As stated above, the chemical reactivity of substrate should be recognized as one of the factors determining regioselectivity in P450-mediated metabolism. However, unless a substrate enters the catalytic cavity of a P450, it cannot be metabolized. Further, even though a substrate can slip into the catalytic cavity of a P450, if the reactive position of the substrate cannot face the heme because of steric hindrance, the P450 will not oxidize this compound at the expected site. Therefore, the three-dimensional structure of the substrate-binding site of this enzyme is a critical factor in the regioselectivity of P450. Several approaches have been used to investigate the active site of P450.

Estimation by Using Chemical Probes

3-Methylcholanthrene- and phenobarbital-inducible forms of P450, especially rat CYP1A1 and CYP2B1, are the isozymes studied most extensively. Elucidation of the catalytic site of these two isozymes has been based on a detailed knowledge of their substrates. That is, from the comparison of the area/depth^2 values of substrates, it is proposed that CYP1A1 metabolizes compounds with planar structures whereas CYP2B1 metabolizes bulky compounds (149–151). Another comparison using length/width values does not distinguish between substrates of CYP1A1 and 2B1 (150). This model is well supported by the specificity of CYP1A1 and 2B1 towards the metabolism of dichlorobiphenyls; CYP1A1 hydroxylates coplanar isomers with no chlorine atom at the *o*-position (2,2′,6,6′), while CYP2B1 favorably metabolizes the nonplanar isomers that have two chloro substituents at *o*-positions (2,2′- and 2,6-dichlorobiphenyl) (142). The intermediate isomers

having a chlorine at one *o*-position and another at a lateral position are substrates for both enzymes, although the activities of CYP2B1 are higher than those of CYP1A1 (142). The same structure-activity relationship has been observed in the hydroxylation of 2,5,2′,5′- and 3,4,3′,4′-tetrachlorobiphenyl isomers by purified CYP1A1 and 2B1 (152).

Jerina and his colleagues have proposed a more advanced hypothesis regarding the active site structure of rat CYP1A1. After an analysis of regiospecificity in the CYP1A1-catalyzed metabolism of benzo[*a*]pyrene and its dihydrodiol derivatives, they suggested that the size of the catalytic site of this P450 is a space equivalent to nine benzene rings (153). This model predicts the regioselectivity and stereoselectivity of the CYP1A1-mediated metabolism of a number of polycyclic aromatic hydrocarbons such as naphthalene (154), anthracene (154), benzo[*a*]anthracene (108), benzo-[*c*]phenanthrene (113), and triphenylene (155). For instance, purified CYP1A1 metabolizes benzo[*c*]phenanthrene to the 5,6-oxide and 3,4-oxide in a ratio of 96:4 (113). When the oxygenation position is fixed and the substrate structure (four benzene rings) is superimposed on the model above (nine benzene rings), the configuration giving 5,6-oxide fits well to the model but the other configuration giving 3,4-oxide does not; it allows overflow of one or two rings to the outside of the cavity (113). On the other hand, Imai et al predicted the substrate binding space of rabbit CYP1A2 to be the size of five benzene rings (156, 157). This model is based on the fact that ligand binding and photolysis of the CYP1A2-CO complex are affected differently by a series of polycyclic aromatic hydrocarbons.

Ortiz de Montellano and his co-workers have utilized the chemical modification of heme to assess the space of the P450 catalytic site with the strategy described below. Phenylhydrazine or the more effective reagent, phenyldiazene, forms a phenyl-iron complex with hemoprotein (158). When this complex is treated with acid, phenyl radical is released from iron and re-binds to one of the four nitrogens of the heme pyrroles (158). In this transfer reaction, the four pyrroles (A-, B-, C-, and D-ring) are not equivalent in their capacity to accept the phenyl radical, because of differences in steric hindrance of each pyrrole ring by amino acids of the P450 peptide. Thus, it is possible to estimate the space of the upper area of heme by analyzing the pyrrole that is modified. This methodology has been applied to purified CYP1A1, and the results showed that the ratio of A-, B-, C-, and D-rings containing *N*-phenyl groups is 2:0:0:1 (159). This means that the pyrrole B- and C-rings of CYP1A1 heme are highly shielded by amino acids, while the A- and D-rings allow interaction with substrate. The space of the upper area of the heme in CYP2B1, 2B2, 2B4, 2B10, 2B11, 2E1, 101 ($P450_{cam}$), and 108 ($P450_{terp}$) has been estimated similarly. Although the B-ring of all of these P450s is highly shielded, the upper spaces of

other pyrroles differ from each other (159–161). From the crystal structure of $P450_{cam}$, the pyrrole B-ring is shown to be completely covered by an α-helical sequence (the so-called I helix), which lies just above the heme (162). This is very consistent with the data above. On the other hand, CYP102 ($P450_{BM-3}$) shows a somewhat different nature from the isozymes described above; the B-ring of this P450 can be modified by a phenyl cation (161). When 2-naphthylhydrazine, 4-(phenyl)phenylhydrazine, and 3,5-dimethylphenylhydrazine are used instead of phenylhydrazine as the aryl cation source, they give the much different ratios of the N-substituted A-, B-, C-, and D-rings of $P450_{cam}$ (163). Modification of the heme in other P450s by these reagents has not yet been reported.

There are many specific substrates for the CYP2D subfamily of P450, and all of these are nitrogen-containing compounds. The comparison of hydroxylation sites led to a hypothesis that the nitrogen atom of the CYP2D P450 substrate plays an essential role in the interaction with P450 (164–167). The fact that the metabolic site of CYP2D P450 substrate is always located 5–7Å away from the basic nitrogen atom suggests that this position may have a function in fixing substrate through the interaction with an amino acid, thought to be aspartic acid 301 (167). Further, the coplanar conformation having a negative electrostatic potential, which is located near the metabolic site, may be the other determinants (168). Prediction of the site of oxidation may be possible by superimposing a chemical structure of a substrate to the template that agrees with the above conditions for the CYP2D P450 substrate. Koymans et al predicted the metabolic site of four compounds, i.e. alfentanil, astemizole, risperidone, and nebivolol, which had not been known as substrates of CYP2D P450, and reported that three of four predicted sites were in fact hydroxylated (168). Similar conclusions were drawn in studies using 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) as probes (169).

Recently, Smith & Jones speculated on the structural requirement for substrates of the CYP2C subfamily (167). They demonstrated the requirement of a functional group, which is capable of forming hydrogen bond with the cytochrome, in the substrate of human CYP2C9. Examples of the functional groups involved are the carboxylate in ibuprofen, naproxen and diciofenac, and the sulfonylurea of tolbutamide. However, CYP2C9 can metabolize benzo[*a*]pyrene (99), which has no such functional group. Furthermore, a question arises whether or not the basic position can be considered as the functional site interacting with P450 amino acid, since CYP2C9 also metabolizes benzphetamine at a high rate (99). The CYP2C subfamily is the most complicated group because there are many isozymes resembling each other in their primary structure, but which differ function-

ally. Perhaps, each CYP2C P450 has a different mechanism to fix the substrate on the catalytic site. Much remains to be clarified on this problem.

Several factors that are required of compounds for interaction with the CYP2E subfamily P450 have been theoretically estimated (170). Thus, an investigation of relationships between the inhibitory potentials of various alcoholic compounds toward aniline hydroxylation and several parameters related to the molecular shapes and/or electronic status of inhibitors has been performed. The best correlation has been found between the lowest unoccupied molecular orbital levels at the α-carbon atom of the inhibitor and its inhibitory potential. The author speculated that electron donation toward the α-carbon may be from the porphirin ring or an amino acid located near the active site. This may be one of the mechanisms by which CYP2E P450 fixes its own substrate. In addition to the electronic features mentioned above, the ratio of area/depth of alcoholic inhibitors is also correlated with their inhibitory action (170).

As mentioned elsewhere, the molecular size of substrates of the CYP3A subfamily P450 varies extensively (8, 167). Large molecules, i.e. cyclosporine (104, 171), erythromycin (44, 172), and FK-506 (173), are metabolized by this family. If a large molecule can bind, then small substrates, such as testosterone, would have greater mobility at the catalytic site. Therefore, the site of oxidation by the CYP3A subfamily may be dictated more by the chemical reactivity of the substrate than may be the case for reactions catalyzed by other families. In this context, CYP3A P450 is known to be the testosterone 6β-hydroxylase enzyme (see Table 1). This position of testosterone is allylic and has greater reactivity than other aliphatic sites. The same examples have been reported by Smith & Jones (167).

Site-Directed Mutagenesis of P450 and Modeling of the Three-Dimensional Structure of P450

X-ray analysis of crystal structure is one of the best ways to resolve the molecular conformation of protein. However, this analysis has only been made for the bacterial P450, $P450_{cam}$ (162, 174–177). Successful crystallization has been reported for $P450_{SCC}$ (178) as well as the bacterial P450s, $P450_{tcrp}$, and $P450_{BM\text{-}3}$ (179), but the details of X-ray analysis were not included in the report. The X-ray data for the $P450_{cam}$-camphor complex gave invaluable information on substrate-P450 interaction. The role of Tyr 96 is most important; it was shown that this amino acid plays a role in holding the camphor molecule through hydrogen bonding between its hydroxyl group and camphor carbonyl (162). Further, other amino acids, i.e. Phe 87, Leu 244, Val 247, and Val 295 are thought to make a hydrophobic environment at the catalytic site (162).

Site-directed mutagenesis is a useful methodology for identifying amino

acids participating in enzymatic reactions. The mutation of Tyr 96 of $P450_{cam}$ to Phe resulted in a decrease in the regioselectivity as well as the hydroxylation efficiency of camphor hydroxylation (30). Changing the substrate from camphor to thiocamphor or camphane, which are not capable of forming hydrogen bonds with Tyr 96, decreases the specificity of wild type $P450_{cam}$ (30). These results clearly indicate the important function of Tyr 96 in fixing camphor to the active site. In addition, a study using the $P450_{cam}$ mutant in which Tyr 96 is changed to Phe demonstrated that both the spin state and the cooperativity between camphor and cation binding are altered by this mutation (180). The mutation of Phe 87 in $P450_{cam}$ to Ala has been reported to cause the enlargement of a space just above the pyrrole D-ring of heme (163). As mutation of Thr 252 of $P450_{cam}$ to Ala or Val causes uncoupling of the catalytic cycle, this residue undoubtedly plays an important role in O_2 utilization (181). X-ray analysis of the above mutant indicated that a solvent molecule is located near the dioxygen binding site, interfering with the access of dioxygen (182). In agreement with this notion, Thr 252 in I helix is highly conserved in P450 enzymes (183, 184). On the other hand, Thr 301 in rabbit CYP2C2 and CYP2C14, which correspond to Thr 252 of $P450_{cam}$, are thought to play a role in substrate binding (185, 186). Similarly, it was reported by site-directed mutagenesis experiments that various amino acid residues of CYP1A2 that are contained in the region (distal region) corresponding to I helix of $P450_{cam}$ affect the catalytic specificity of this P450 (187). The amino acid residues of $P450_{cam}$ participating in the interaction with the partner enzyme, putidaredoxin reductase, have been studied (188).

Group-to-group alignment of sequences of various mammalian P450s suggests that the catalytic site consists of six separate regions [substrate recognition site (SRS)-1 to (SRS)-6] in agreement with data from the crystal structure of $P450_{cam}$ (189). An increasing number of studies have identified the amino acid residues affecting the catalytic activity of mammalian P450s. A variant CYP2B2 cDNA has been isolated from the λgt 11 library of untreated rat liver, which has mutations equivalent to amino acid substitutions of Leu 58 and Ile 114 (190). Studies using the expressed recombinant CYP2B1 and 2B2, which have mutation(s) at the above sites, revealed that an enzyme with double mutation shows regioselectivity in testosterone hydroxylation that is quite different from that of the native form (190). From these results, the authors speculated that these two amino acids, located in the N-terminal half, affect substrate orientation at the catalytic site. Also, the above recombinant forms of P450 were shown to exhibit metabolic patterns towards 7,12-dimethylbenz[*a*]anthracene and benzo[*a*]pyrene that differed from those of the native form (191). A variant of CYP2B1 has a mutation at Gly 478, and this form shows a decreased ability to hydroxylate

androstenedione at the 16β-position (192, 193). The result suggests that this amino acid residue also affects the substrate orientation.

The residue of Asp 251 in $P450_{cam}$ and the corresponding residues (Asp or Glu) in other P450s are one of the highly conserved amino acids (183). Based on the crystal structure of $P450_{cam}$, this amino acid seems to be involved in the heme cavity. This possibility was examined by using some mutant CYP1A2s, in which the amino acid residues of Glu318 and the following Thr-rich sequences were changed. The results showed that ligand binding, ligand-induced spin state change, and catalytic turnover are affected by introducing mutations (194–196). These workers also made other mutants of CYP1A2 and reported that ionic amino acid residues such as Lys 94, Lys 99, Lys 105, Lys 440, Lys 453, Lys 455, Lys 463, and Arg 135–137 may have a function in electron transfer between CYP1A2 and reductase (187, 197). One (SRS-3) of the proposed regions of the catalytic site is thought to contain a series of positively charge amino acids. However, the amino acids corresponding to the above region, i.e. Lys 250, Arg 251, and Lys 253, do not appear to be important in the intrinsic catalytic activity of CYP1A2, because the mutants that have neutral amino acids instead of the ionic amino acids mentioned above showed higher activities than did the native enzyme (198).

Two mouse P450s belonging to the Cyp2a subfamily, P450coh and $P450_{15\alpha}$, are closely related proteins as they differ in only 11 amino acid residues of the sequence (199). In spite of the similarity of structure, the above two enzymes show different catalytic natures; P450coh catalyzes coumarin 7-hydroxylation while $P450_{15\alpha}$ effects 15α-hydroxylation of Δ^4 3-ketosteroids. Negishi and his colleagues focussed on these points and studied the amino acid residues that reflect differences in hydroxylation specificity. For this purpose, hydroxylase activity was assayed in mutants of P450coh in which the 11 residues were each changed to the $P450_{15\alpha}$ type sequence. The results indicated that the three residues, Val 117, Phe 209, and Met 365, were important determinants of P450coh specificity (200). In addition, it was evident that Phe 209 has an important role in the hydroxylation of the 15α-position of steroids. Only the substitution at Phe 209 of P450coh by Leu ($P450_{15\alpha}$-type amino acid) resulted in a significant increase in this metabolic activity (200). A series of P450coh mutants in which Phe 209 was replaced with different amino acids were constructed, and their spectral and catalytic properties, and susceptibility to inhibition by coumarin derivatives, were examined (201, 202). Some of these mutant P450s are active in corticosterone 15α-hydroxylation, a reaction not catalyzed by wild type P450coh (203).

Similar to the above example of mouse Cyp2a P450s, rabbit CYP2C4 and 2C5 are highly related (95% similarity) but are functionally distinct

enzymes. That is, only the latter P450 effectively hydroxylates progesterone at the 21-position. The progesterone binding domain of CYP2C5 has been estimated to be the region between the 113th and the 118th residues by constructing chimera enzymes (204). Further study by single mutation indicated that Val 113 of CYP2C5 plays a critical role in defining the substrate specificity of this P450 (205, 206). There are allelic variants of human CYP2C9 and CYP2C18. The cDNAs coding these proteins or the mutagenized cDNA were expressed and the catalytic properties were compared by using mephenytoin (207), phenytoin (208), tolbutamide (208), and warfarin (209) as the substrates. A number of CYP2A1 and 2A2 mutants have been constructed and the capacities of the expressed P450s in testosterone hydroxylation have been determined (210). In addition, the functional domains of steroidogenetic P450s such as aromatase (211–214), $P450_{17\alpha}$ (215), $P450_{21\alpha}$ (216, 217), and $P450_{11\beta}$ (218) have been inferred by site-directed mutagenesis.

SUMMARY AND FUTURE DIRECTIONS

Stereochemical aspects of drug metabolism are the object of renewed interest as investigators seek to determine which chiral drugs can be marketed and administered as a single enantiomer or as the racemate. These concerns and a general interest in protein structure and function have resulted in numerous regio- and stereochemical investigations of P450 isozymes. The theoretical basis of regioselective drug metabolism was first considered in terms of the contributions of the frontier orbital densities (HOMO/HOMO-1) of the substrates and the relative ionization potentials of the resulting radicals. The binding of the substrate to a chiral macrobiomolecule results naturally in regioselective recognition. The binding and recognition factors contribute preferentially to the regioselective reaction. The present review has focussed mainly on the regio- and stereoselectivity of P450.

Current advances in computational chemistry allow us to simulate the tertiary structure of P450 enzymes. In this approach, the modeling has been based on the crystal structure of $P450_{cam}$ (162) and on the sequence alignment between the target P450 and $P450_{cam}$ (183, 184). Alignment of the sequence of $P450_{scc}$ revealed 69 identical residues and many highly conserved regions with the sequence in $P450_{cam}$ (219). The model of $P450_{17\alpha}$ suggested the possibility of two models for the binding of steroid substrates at the active site (220). Further work in this area using physical and molecular techniques will produce more refined structural models and may enable us to predict the regioselectivity of P450s by evaluating interactions with hydrophobic, hydrogen, and ionic bonds.

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