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Correlation of Human Cytochrome P4502C Substrate Specificities with Primary Structure: Warfarin as a Probe

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SUMMARY

The regio- and stereoselectivity of warfarin metabolism have been used to assess structure-function relationships of human P4502C subfamily members. Metabolism was investigated using a yeast cDNA expression system in which full length cDNAs for P4502C8, -2C9 (alleles Arg^{144} Tyr^{358} Ile^{359} Gly^{417} and Arg^{144} Tyr^{358} Leu^{359} Gly^{417}), -2C18 (alleles Thr^{385} and Met^{385}), and -2C19 were expressed. Additionally, two mutations reported in other P4502C9/2C10 alleles were individually introduced into P4502C9 by site-directed mutagenesis, to yield Cys^{144} Tyr^{358} Ile^{359} Gly^{417}, Arg^{144} Tyr^{358} Ile^{359} Asp^{417}, and Arg^{144} Cys^{358} Ile^{359} Gly^{417}, which were expressed in yeast; their ability to metabolize warfarin was then studied. Warfarin metabolism by purified preparations of P4502C9 allele Arg^{144} Tyr^{358} Ile^{359} Gly^{417} and its Leu^{359} mutant was also investigated in reconstituted systems. Both alleles of P4502C18 were regioselective for 4′-hydroxywarfarin, without any significant stereoselectivity. Both also metabolized warfarin at the 6-position, but to a lesser extent, and metabolism at this site was stereoselective for (*R*)-warfarin. P4502C8 metabolized warfarin at the 7-position and was stereospecific for (*R*)-warfarin. It also metabolized warfarin to a lesser extent at the 4′-position, and metabolism at this site was stereoselective for (*R*)-warfarin. P4502C19 was regioselective for 6- and 8-hydroxywarfarin and was stereoselective for (*R*)-warfarin. The highly conservative mutation of Ile^{359} to Leu^{359} in P4502C9 profoundly altered the regio- and stereoselectivity of warfarin metabolism, from regioselective for 7-hydroxywarfarin, with stereospecificity for (*S*)-warfarin, to regioselective for 4′-hydroxywarfarin, with stereoselectivity for (*R*)-warfarin, which was confirmed in a reconstituted system using purified recombinant enzymes. In contrast, individual mutations of P4502C9 of Arg^{144} to Cys, Tyr^{358} to Cys, and Gly^{417} to Asp did not markedly affect the regio- or stereoselectivity of warfarin metabolism, although the overall rates of warfarin metabolism were apparently increased by these changes. We conclude that residue 359 is at the substrate binding site of P4502C9, whereas residues 144, 358, and 417, and residue 385 of P4502C18, are not.

The human P4502C subfamily, comprising five currently identified members together with allelic variants, plays an important role in the metabolism and associated polymorphisms of a variety of drugs. Some confirmed P4502C drug substrates are mephenytoin, hexobarbital, benzo[*a*]pyrene, tolbutamide, warfarin, phenytoin, and tienilic acid (1–8), whereas others such as retinol, diazepam, nirvanol, ethotoin, mephobarbital, methsuximide, and phensuximide have been proposed as putative substrates (as summarized in Ref. 5). In the cases of at least mephenytoin, warfarin, and hexobarbital, metabolism is stereoselective (1, 2). Human liver P4502C subfamily members that have been sequenced include several allelic variants of P4502C8 (9–12), allelic variants of P4502C9 (10, 11, 13–15), P4502C10 (11, 16), two alleles of P4502C18, and P4502C19 (15). A cDNA classified as P4502C10 varies in the coding region from one P4502C9 by two amino acids and could represent an allele of P4502C9. However, at present it has been classified as a separate gene on the basis of a divergent 3′ end.

The availability of full length cDNAs for members of the P4502C subfamily and cDNA expression systems provides an opportunity to conduct structure-function studies to gain insight into which specific amino acids or sequences are involved in substrate binding and positioning for reaction. One ideal substrate for such studies is the anticoagulant drug warfarin, which is metabolized by a variety of different P450s at several aromatic and aliphatic carbons (2, 17–19). Warfarin has been resolved into its *R*- and *S*-enantiomers, thus permitting assessments of P450 metabolic stereo- and regioselectivity (20).

In this paper the regio- and stereoselectivity of warfarin metabolism by members of the human liver P4502C subfamily are described. A number of alleles of P4502C9 were constructed by site-directed mutagenesis. Metabolism was investigated using a yeast cDNA expression system in which full-length cDNAs for P4502C8, P4502C9 (alleles Arg^{144} Tyr^{358} Ile^{359} Gly^{417},

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ABBREVIATIONS: P450, cytochrome P450; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SRS, substrate recognition site.

Arg[144] Tyr[358] Leu[359] Gly[417], and Cys[144] Tyr[358] Ile[359] Gly[417]), P4502C18 (alleles Thr[385] and Met[385]), and P4502C19 were expressed. P4502C10 varies from P4502C9 (Arg[144] Tyr[358] Ile[359] Gly[417] allele) by two amino acids (Cys[358] and Asp[417]). Each of these two amino acids was also introduced individually into P4502C9 and the metabolism of warfarin by these mutants was studied. In addition, the metabolism of warfarin by purified preparations of P4502C9 alleles Arg[144] Tyr[358] Ile[359] Gly[417] and Arg[144] Tyr[358] Leu[359] Gly[417] was investigated in a reconstituted enzyme system. The metabolic data are used to develop structure-function relationships for the P4502C subfamily.

Experimental Procedures

Materials. Racemic warfarin from Calbiochem (Los Angeles, CA) was resolved by fractional crystallization (21) into optically pure (*R*)-($[\alpha]_D$ = +149) and (*S*)-warfarin ($[\alpha]_D$ = −149.7). The warfarin metabolites 4′-, 6-, 7-, and 8-hydroxywarfarin were synthesized by modification (22) of previously reported methods (23–25). Metabolites were characterized by UV spectra and melting points. NADPH-P450 reductase was purified to electrophoretic homogeneity from the liver of phenobarbital-treated rats by published procedures (26).

The strategy for expressing full length cDNAs for P4502C8 (allele Thr[130] Ile[264]), P4502C9 (alleles Arg[144] Tyr[358] Leu[359] Gly[417] and Arg[144] Tyr[358] Ile[359] Gly[417]), P4502C18 (alleles Thr[385] and Met[385]), and P4502C19 in yeast has been described previously (27). Yeast microsomes were prepared from transformed cells isolated at midlogarithmic phase as described previously (28). Protein concentrations were determined by the method of Bradford (29). P450 concentrations of yeast microsomes were determined by dithionite-reduced carbon monoxide difference spectra by the method of Omura and Sato (30), using an extinction coefficient of 91 mM^{-1} cm^{-1}. Spectra were recorded in 100 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol and 0.1 mM EDTA. The sample was reduced with a few milligrams of sodium dithiononite and was divided between two cuvettes. The sample was bubbled with CO for 60 sec, and the difference spectrum was recorded from 395 to 550 nm, in a DW2000 spectrophotometer (SLM Aminco, Urbana, IL), with Spectrum processor software. The spectrum was recorded with both cuvettes filled with buffer alone, and this minimal spectrum, which corrected for any instrument variations over this wavelength, was subtracted using the Spectrum processor software. Recombinant proteins for two of the P4502C9 alleles (Arg[144] Tyr[358] Leu[359] Gly[417] and Arg[144] Tyr[358] Ile[359] Gly[417]) were also purified from yeast microsomes for reconstitution studies, using octylamino-Sepharose and hydroxyapatite chromatography (27).

Site-directed mutagenesis and expression of P4502C9 alleles. The cDNA for P4502C9 (clone 65) (Arg[144] Tyr[358] Ile[359] Gly[417]) was isolated by *Hind*III digestion, ligated into M13, and screened by restriction analysis for the proper orientation. Single-stranded DNA templates were then prepared. Site-directed mutagenesis reactions were performed using an oligonucleotide-directed *in vitro* mutagenesis kit (Amersham Corp.), based on the methodology of Eckstein and coworkers (31, 32). Oligonucleotides for site-directed mutagenesis were synthesized using an Applied Biosystems model 380B DNA synthesizer. To form the Cys[144] Tyr[358] Ile[359] Gly[417] allele, the oligonucleotide Cys[144]F (5′-ATTGAGGACTGTGTTCAAGA-3′; the introduced mutation is underlined) was used. P4502C10 differs from P4502C9-Arg[144] Tyr[358] Ile[359] Gly[417] by Cys[358] and Asp[417] substitutions. To individually introduce the Cys[358] and Asp[417] substitutions, MP8.358F (5′-CCAGAGATGCATTGACCTTC-3′) and MP8.417F (5′-GGATGAAGGTGACAATTTTTA-3′) were used in two separate site-directed mutagenesis reactions. All mutant cDNAs were verified by dideoxy sequencing using Sequenase kits (United States Biochemical Corp., Cleveland, OH). The mutants and their relationship to known alleles of P4502C9/2C10 are shown in Table 1.

TABLE 1
Relationship of P4502C9/10 alleles and mutants studied

P4502C9 alleles expressed[a]	Clone	Amino acid substitutions				Ref.
		144	358	359	417	
Cys[144] Ile[359]	IICI	Cys	Tyr	Ile	Gly	10
	Hum2, MP4, clone 65	Arg	Tyr	Ile	Gly	8, 11, 14
Leu[359]	Clone 25	Arg	Tyr	Leu	Gly	8
	MP8 (2C10)[b]	Arg	Cys	Ile	Asp	11
Cys[358]	Mutant[b]	Arg	Cys	Ile	Gly	
Asp[417]	Mutant[b]	Arg	Tyr	Ile	Asp	

[a] The first column refers to the P4502C9 allele or P4502C9 mutant expressed in the yeast cDNA expression system in the present study. Subsequent columns show the relationship to published P4502C9/2C10 alleles.
[b] Each of the two amino acid changes in P4502C10 (MP8) was substituted individually into clone 65.

The mutant inserts were then digested from the vectors with *Hind*III and ligated to the pAAH5 vector. The recombinant plasmids were isolated from *Escherichia coli* DH5 cells using Qiagen plasmid purification kits (Qiagen, Inc., Studio City, CA). *Saccharomyces cerevisiae* 334 cells were grown in YPD medium [2% bactopeptone (w/v), 1% yeast extract (w/v, Difco) and 2% dextrose (w/v)] to late logarithmic phase and were transformed with the plasmid using the lithium acetate method, as described previously (28). Recombinant yeast microsomes were then prepared as described above.

Warfarin hydroxylase assays. The regio- and stereoselectivities of warfarin metabolism by human P4502C forms expressed in yeast or by purified and reconstituted P4502C forms were determined by modifications of published procedures (33). All assays were run in triplicate unless otherwise indicated. In all cases control experiments were conducted with yeast microsomes without expressed P4502C forms. In no case were warfarin metabolites detected. In some cases limited availability of materials prevented triplicate analyses. Yeast microsomes (4 mg/ml) were fortified with exogeneous rat liver NADPH-P450 reductase (0.5 μM) in 50 mM HEPES buffer, pH 7.4, containing 0.01 mM EDTA and 0.15 mM $MgCl_2$ and were incubated at 37° for 3 min. Warfarin [(*R*)- or (*S*)-, 2.9 mM] and water (to bring total volume to 0.25 ml) were added and the mixture was incubated at 37° for 1 min. The reaction was initiated with NADPH (2.4 mM) and incubated for 30 min with shaking, before being terminated by immersion in an ice bath. For reconstituted systems, P4502C (0.25 μM), rat liver NADPH-P450 reductase (0.75 μM), and L-α-dilauroylglyceryl-3-phosphorylcholine (0.1 mg/ml) in 50 mM HEPES buffer, pH 7.4, and water (to bring total volume to 0.25 ml) were incubated with warfarin [(*R*)- or (*S*)-, 2.9 mM] at 37° for 1 min. NADPH (2.4 mM) was added to initiate the reaction, which was continued for 30 min with shaking before being terminated by immersion in an ice bath. Ice-cold water (0.25 ml) was added to the microsomal and reconstituted reaction systems, and the reaction mixture was filtered through a pad of filters (Millipore 1.2, 0.65, and 0.22 μm) in a 13-mm Swinnex filter unit. The filtered solution was analyzed by high performance liquid chromatography on a Waters Associates C18 Novapak column (33). Metabolites were quantitated by the use of synthetic external standards, after resolution with a gradient of ammonium acetate (pH 4.85) and acetonitrile, with detection at 313 nm.

Statistical analyses. Rates of warfarin metabolism were compared using Student's *t* test (significant at $p < 0.05$).

Results

Carbon monoxide difference spectra of recombinant yeast microsomes revealed that the level of expression of the recombinant P4502C proteins in yeast microsomes varied from 13 to 75 pmol/mg of protein, except for P4502C19, which was expressed poorly (<6 pmol/mg). However, the more sensitive technique of Western blot analysis had previously shown that

P4502C19 is also expressed in these recombinant yeasts at a level of approximately ≤6 pmol/mg (27). The values for P450 content of the recombinant yeast microsomes used in the present study are shown in the figure legends. The regio- and stereoselectivities of warfarin metabolism by yeast microsomal preparations with expressed P4502C protein, in the presence of exogenous rat liver NADPH-P450 reductase, are shown in Figs. 1–4 and of that by reconstituted purified recombinant P4502C9 alleles in Fig. 5.

The Thr^{385} and Met^{385} alleles of P4502C18 expressed essentially identical regio- and stereoselectivities for warfarin metabolism (Fig. 1), although the overall rates of metabolism were slightly, but statistically significantly, higher for the Met^{385} allele. Both alleles were regioselective for 4′-hydroxywarfarin, without any significant stereoselectivity. Both also metabolized warfarin at the 6-position, but to a lesser extent, and metabolism at this site was stereoselective for (*R*)-warfarin. P4502C8 (Fig. 2) metabolized warfarin at the 7-position and was stereospecific for (*R*)-warfarin. It also metabolized warfarin to a lesser extent at the 4′-position, and metabolism at this site was stereoselective for (*R*)-warfarin. P4502C19 (Fig. 3) was regioselective for 6- and 8-hydroxywarfarin and stereoselective for (*R*)-warfarin.

The regio- and stereoselectivities of warfarin metabolism by the P4502C9 alleles are shown in Fig. 4. The highly conservative mutation of Ile^{359} to Leu^{359} profoundly altered the regio- and stereoselectivity of warfarin metabolism, from regioselective for 7-hydroxywarfarin and stereospecific for (*S*)-warfarin to regioselective for 4′-hydroxywarfarin and stereoselective for (*R*)-warfarin. This result was closely duplicated with an additional batch of yeast expressing these two alleles (data not shown), thus confirming the effect of this single amino acid substitution on the regio- and stereoselectivity of warfarin metabolism. The differences in the metabolism of warfarin by these allelic forms of P4502C9 were also confirmed in a reconstituted system using purified recombinant enzymes (Fig. 5). The regio- and stereoselectivities of the purified P4502C9 alleles essentially reflected those in recombinant yeast microsomes, i.e., 7-hydroxylation of (*S*)-warfarin by Ile^{359} and 4′-hydroxylation of (*R*)-warfarin by Leu^{359} alleles. However, the relative rates of formation of the various metabolites in reconstituted systems differed somewhat from those in microsomes. It should be noted that, because of the limited availability of the purified recombinant enzymes, the reconstituted system constituent ratios were based on reconstitution conditions that optimized tolbutamide metabolism by P4502C9 purified from human liver in earlier studies (27). In contrast to the major differences in warfarin metabolism for the Ile^{359} and Leu^{359} P4502C9 alleles, individual mutations of P4502C9 Arg^{144} to Cys, Tyr^{358} to Cys, and Gly^{417} to Asp, seen in other previously reported allelic variants of P4502C9/2C10 (10, 11), did not markedly affect the regio- or stereoselectivity of warfarin metabolism (Fig. 4), although the overall rates of warfarin metabolism were significantly increased by these changes.

Discussion

The various P4502C subfamily members and alleles investigated in this study all metabolized warfarin, yielding patterns of regio- and stereoselectivity that permit structure-function assessments. Comparisons of the warfarin hydroxylase activities of P4502C forms, particularly those that vary in only one amino acid residue, identify those residues that influence substrate specificity, either directly by being at the substrate binding site or indirectly by influencing the three-dimensional structure of that site.

Recently, Gotoh (34) has proposed six putative SRSs for the P4502 family, based on alignment of P4502 family member sequences with that of bacterial P450101 and on comparisons of hydropathy indices and secondary structure information. The substrate binding site of P450101 has been identified by X-ray crystallography (35). In addition, all published point mutations and chimeric fragments that significantly affect substrate specificities of P4502 forms were included in the SRS assignment process by Gotoh (34), so as to either fall within or overlap with one of the sites. Human P4502C forms included in this analysis were P4502C8, -2C9, -2C18, and -2C19 (34).

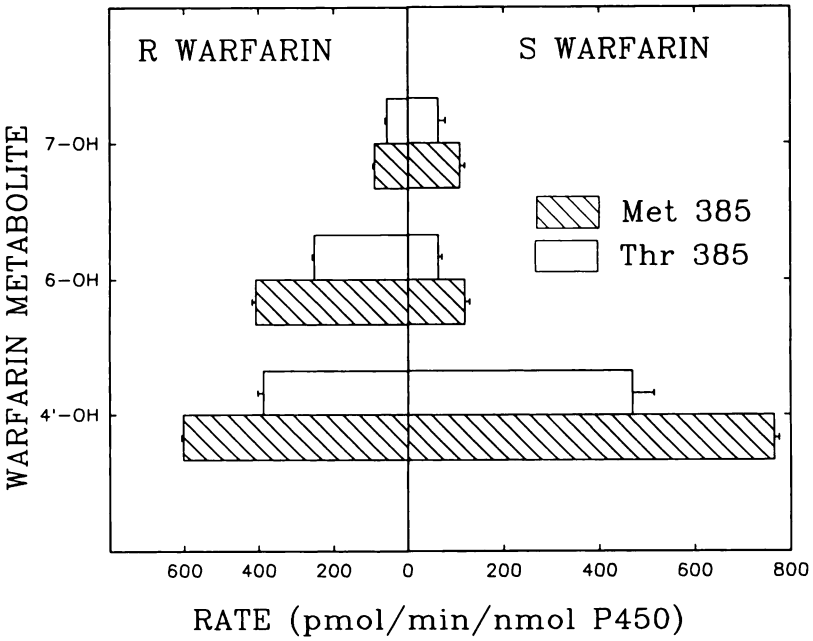

Fig. 1. Rates of formation of the 4′-hydroxy, 6-hydroxy, and 7-hydroxy warfarin metabolites from (*R*)- and (*S*)-warfarin catalyzed by recombinant human liver P4502C18 allelic variants (Met^{385} and Thr^{385}) expressed in yeast microsomes. Microsomal P4502C18 concentrations were 46.3 and 27.0 pmol/mg of microsomal protein for the Thr^{385} and Met^{385} alleles, respectively (corrected for constitutive yeast P450, 6.7 pmol/mg of protein), with the CO maximum at 451.4 nm. Other experimental conditions are described in Experimental Procedures. Rates are mean ± standard deviation (three experiments).

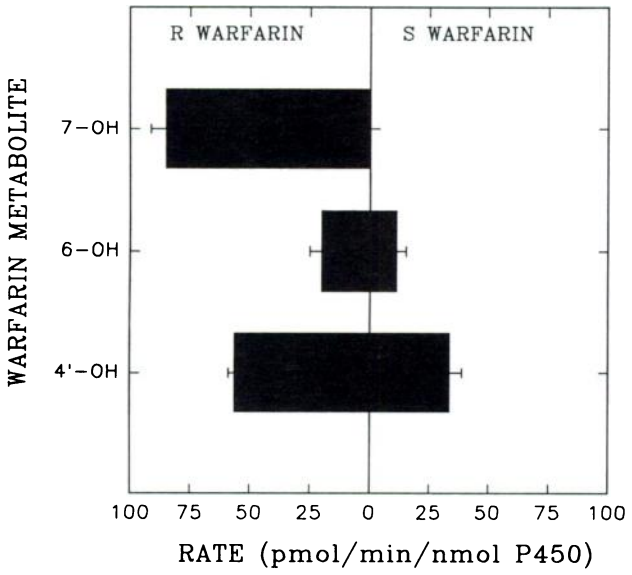

Fig. 2. Rates of formation of the 4′-hydroxy, 6-hydroxy, and 7-hydroxy metabolites from (*R*)- and (*S*)-warfarin catalyzed by recombinant human liver P4502C8 expressed in yeast microsomes. The microsomal P4502C8 concentration was 75 pmol/mg of microsomal protein (corrected for constitutive yeast P450), with the CO maximum at 451.5 nm. Other experimental conditions are described in Experimental Procedures. Rates are mean ± standard deviation (three experiments).

Site-directed mutations and use of chimeric fragments do not unambiguously prove that the substrate binding site residues of P4502C members fall within these SRSs. However, if a P4502C9 residue that influences substrate specificity falls within one of these SRSs, its putative assignment as a substrate binding site residue in the current study is at least consistent with other published results.

In the present study, comparisons of the five P4502C9 alleles provide the greatest insight into the role of specific amino acid residues in P4502C substrate specificities. Based on the virtual absence of any effect of the nonconservative individual mutations of Arg^{144} to Cys, Tyr^{358} to Cys, and Gly^{417} to Asp, on the stereo- and regioselectivity of warfarin metabolism, we conclude that these residues play little or no role in the substrate binding site architecture of P4502C9. Consistent with this conclusion, none of these residues falls within the putative SRSs assigned by Gotoh (34). However, all of these mutations do significantly, and to a similar extent, appear to increase the overall rate of warfarin metabolism, thus confounding these observations. In view of the diversity of the nature of these mutations, i.e., loss of positive charge, gain of negative charge, and loss of aromaticity, it is difficult to propose an all-embracing mechanism for these rate increases.

In contrast to the effects of the mutations described above, the highly conservative mutation of P4502C9 Ile^{359} to Leu produced profound effects on the stereo- and regioselectivity of warfarin metabolism, principally altering regioselectivity for 7-hydroxylation of warfarin, with stereoselectivity for (*S*)-warfarin, to regioselectivity for 4′-hydroxylation of (*R*)-warfarin. This result was found with two different batches of yeast expressing these two alleles. Additionally, comparisons of the stereo- and regioselectivities of warfarin metabolism by the corresponding purified and reconstituted alleles also confirmed these results. The prominent role of residue 359 in the warfarin hydroxylase activity of P4502C9 is consistent with its location within putative SRS 5, as proposed by Gotoh (34). In addition, other studies have shown that mutation of P4502C9 Ile^{359} to Leu decreased the turnover number of the enzyme for tolbutamide (27). Thus, whereas the Ile^{359} to Leu mutation markedly affected the stereo- and regioselectivity of warfarin metabolism by P4502C9, it affected tolbutamide metabolism differently, decreasing its metabolism (27). Clearly, a point mutation can differentially affect the interaction between substrates and their P450 binding site. Presumably, amino acid residues within the substrate binding site of P4502C9 do not all interact with all substrates to the same extent. Thus, mutation of a particular residue within a substrate binding site could differently affect the metabolism of two substrates. The fact that a conservative mutation at residue 359 (within SRS 5) so markedly changes substrate selectivity, whereas a nonconservative mutation at residue 358 (just outside the proposed SRS) is virtually without effect, strongly supports the assignment of SRS 5 (34).

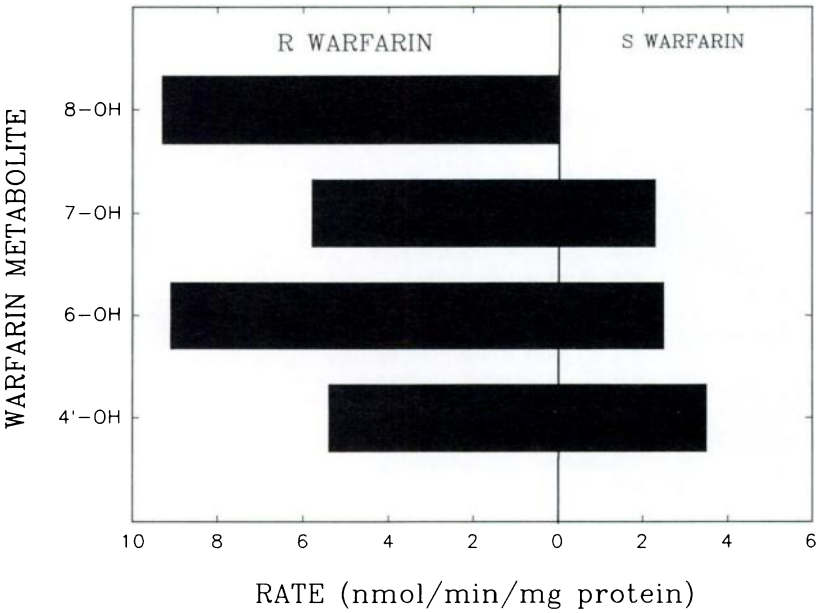

Fig. 3. Rates of formation of the 4′-hydroxy-, 6-hydroxy-, 7-hydroxy-, and 8-hydroxywarfarin metabolites from (*R*)- and (*S*)-warfarin catalyzed by recombinant human liver P4502C19. Other experimental conditions are described in Experimental Procedures. Recombinant P450 concentrations were too low to be determined spectrally (~6 pmol/mg of protein), with the CO maximum at 450 nm. Rates are averages of duplicate experiments.

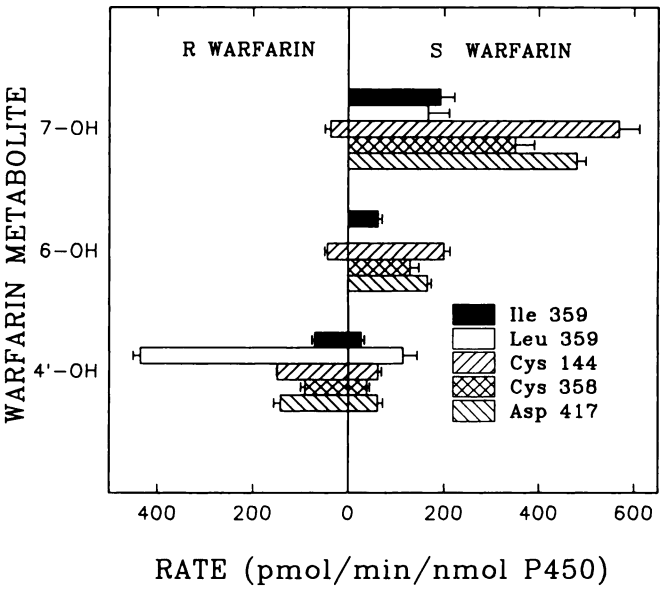

Fig. 4. Rates of formation of the 4′-hydroxy, 6-hydroxy, and 7-hydroxy metabolites from (*R*)- and (*S*)-warfarin catalyzed by recombinant human liver P4502C9/2C10 allelic variants [Arg^{144} Tyr^{358} Ile^{359} Gly^{417} (*Ile 359*), Arg^{144} Tyr^{358} Leu^{359} Gly^{417} (*Leu 359*), Cys^{144} Tyr^{358} Ile^{359} Gly^{417} (*Cys 144*), Arg^{144} Cys^{358} Ile^{359} Gly^{417} (*Cys 358*), and Arg^{144} Tyr^{358} Ile^{359} Asp^{417} (*Asp 417*)] expressed individually in yeast microsomes. Microsomal P4502C9/2C10 concentrations were 48.3, 13.3, 53.0, 32.0, and 35.2 pmol/mg of microsomal protein (corrected for constitutive yeast P450) for the Ile^{359}, Leu^{359}, Cys^{144}, Cys^{358}, and Asp^{417} alleles, respectively, with CO maxima of 450, 450.2, 450.5, 450.7, and 450.7 nm, respectively. Other experimental conditions are described in Experimental Procedures. Rates are mean ± standard deviation (three experiments).

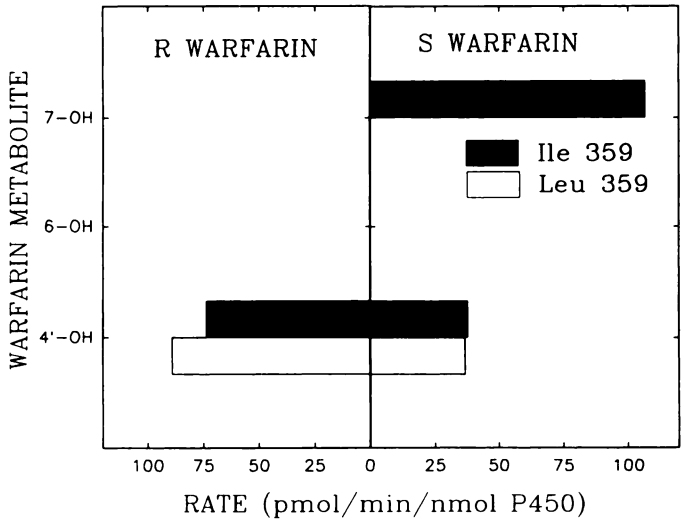

Fig. 5. Rates of formation of the 4′-hydroxy and 7-hydroxy metabolites from (*R*)- and (*S*)-warfarin catalyzed by the purified recombinant human liver P4502C9 proteins Arg^{144} Tyr^{358} Ile^{359} Gly^{417} (*Ile 359*) and Arg^{144} Tyr^{358} Leu^{359} Gly^{417} (*Leu 359*) in a reconstituted system. Other experimental conditions are described in Experimental Procedures. Rates are averages of duplicate experiments.

The P4502C18 Met^{385} and Thr^{385} alleles exhibited the same regio- and stereoselectivities for warfarin metabolism, implying that residue 385, which is not part of a putative SRS (29), plays little or no role in the substrate binding site of P4502C18. In contrast, however, the Thr^{385} allele has a high turnover for the metabolism of tolbutamide, whereas the Met^{385} allele has much lower activity (27). This difference suggests that mutation of an amino acid residue that does not reside in a putative substrate binding site of a P450 can differently affect the metabolism of two substrates by that P450.

P4502C8 and -2C9 exhibited markedly different substrate specificities. Most of the known alleles of P4502C9 metabolized warfarin at the 7-position and were stereoselective for (*S*)-warfarin. In contrast, P4502C8 yielded 7-hydroxywarfarin but was stereospecific for (*R*)-warfarin. These two forms, P4502C8 and -2C9, have only 77% amino acid similarities (10), and thus their observed differences in warfarin hydroxylase stereoselectivities are not surprising. It is surprising, however, that these two P450s, with 23% difference in primary sequence, do in fact metabolize the same substrate, warfarin, at essentially the same sites (but with different enantiomers having differing turnovers). This is a good illustration of the substrate specificity overlaps that occur among some P450s and supports the hypothesis that only a few amino acid residues in P450s govern catalytic specificity (36).

Recent studies by Rettie *et al.* (2) examined the relative rates of formation of 7-hydroxywarfarin by expressed forms of 11 human cDNAs, including P4502C8 and one allelic variant of P4502C9 (Cys^{144} Tyr^{358} Ile^{359} Gly^{417}). P4502C9 had a higher rate of 7-hydroxylation (stereospecific for the *S*-enantiomer of warfarin) than did P4501A2, P4503A4, or any of the other P450s studied. They did not examine the effect of allelic variations on catalytic activity. Our data are consistent with their hypothesis that P4502C9 is a principal enzyme metabolizing the 7-hydroxylation of the *S*-enantiomer of warfarin.

In summary, the members of the P4502C subfamily exhibited marked differences in stereo- and regioselectivity of warfarin metabolism. In addition, site-directed mutagenesis studies examined the role of certain amino acid residues present in a number of allelic variants of P4502C9. Most allelic variants of P4502C9 stereoselectively metabolized (*S*)-warfarin at the 7-position. However, the mutation of a single amino acid (Ile^{359} to Leu) altered both the stereo- and regioselectivity of warfarin metabolism. Mutation of putative substrate binding site residues of P4502C9 differentially affected the metabolism of different substrates [e.g., (*R*)- and (*S*)-warfarin and tolbutamide]. The regio- and stereoselectivity of warfarin metabolism by the P4502C subfamily suggest that this substrate is a good candidate for further defining substrate binding sites in future studies.

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