

Midazolam Oxidation by Cytochrome P450 3A4 and Active-Site Mutants: an Evaluation of Multiple Binding Sites and of the Metabolic Pathway That Leads to Enzyme Inactivation

KISHORE K. KHAN, YOU QUN HE, TAMMY L. DOMANSKI, and JAMES R. HALPERT

Department of Pharmacology and Toxicology, The University of Texas Medical Branch, Galveston, Texas.

Received July 13, 2001; accepted November 28, 2001

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Midazolam (MDZ) oxidation by recombinant CYP3A4 purified from *Escherichia coli* and 30 mutants generated at 15 different substrate recognition site positions has been studied to determine the role of individual residues in regioselectivity and to investigate the possible existence of multiple binding sites. Initial results showed that oxidation of MDZ by CYP3A4 causes time- and concentration-dependent enzyme inactivation with K_i and k_{inact} values of 5.8 μ M and 0.15 min^{-1} , respectively. The different time courses of MDZ hydroxylation by mutants that predominantly formed 1'-OH MDZ as opposed to 4-OH MDZ provided strong evidence that the 1'-OH MDZ pathway leads to CYP3A4 inactivation. Correlational analysis of 1'-OH formation versus 4-OH formation by the mutants supports the inference that the two metabolites result from the binding of MDZ at two

separate sites. Thus, substitution of residues Phe-108, Ile-120, Ile-301, Phe-304, and Thr-309 with a larger amino acid caused an increase in the ratio of 1'-OH/4-OH MDZ formation, whereas substitution of residues Ser-119, Ile-120, Leu-210, Phe-304, Ala-305, Tyr-307, and Thr-309 with a smaller amino acid decreased this ratio. Kinetic analyses of nine key mutants revealed that the alteration in regioselectivity is caused by a change in kinetic parameters (V_{max} and K_M) for the formation of both metabolites in most cases. The study revealed the role of various active-site residues in the regioselectivity of MDZ oxidation, identified the metabolic pathway that leads to enzyme inactivation, and provided an indication that the two proposed MDZ binding sites in CYP3A4 may be partially overlapping.

Midazolam (MDZ) is one of the most commonly used drugs for sedation in emergency rooms (see Nordt and Clark, 1997 and references therein). It is also used as a safe and effective drug for the treatment of generalized seizure, status epilepticus, and acute agitation. The biotransformation of MDZ is mediated by at least three different CYP3A enzymes: 3A4, 3A5, and 3A7 (Gorski et al., 1994; Kuehl et al., 2001). Although CYP3A7 is predominantly expressed in fetal tissues, CYP3A4 and CYP3A5 represent the majority of the total hepatic and intestinal P450 content in adults (Guengerich, 1995). These enzymes are of particular clinical significance because of their ability to metabolize a large number of therapeutic agents of very diverse structures (Guengerich, 1995). Moreover, intestinal CYP3A accounts for significant first-pass metabolism of ingested drugs. Because of the large number of therapeutic agents that alter CYP3A expression or

activity, a significant potential for drug-drug interactions exists (Fuhr et al., 1996). In particular, CYP3A4 is known to exhibit both homotropic and heterotropic cooperativity, which could influence drug metabolism and excretion or bioactivation (Schwab et al., 1988; Shou et al., 1994, 2001; Harlow and Halpert 1997, 1998; Domanski et al., 1998, 2000; Korzekwa et al., 1998).

In recent years, MDZ has emerged as one of the best in vivo probes for prediction of CYP3A activity (Thummel and Wilkinson, 1998). MDZ can be administered both orally and intravenously, which can provide a measure of CYP3A activity relative to intestinal and hepatic metabolism, respectively. Furthermore, according to in vitro studies, MDZ is not subject to P-glycoprotein-mediated transport across the intestinal epithelium (Kim et al., 1999). Additionally, a difference in the regioselectivity of MDZ metabolism at lower concentrations can be used to discriminate among individual subjects with or without CYP3A5, because CYP3A5 shows a

This work was supported by National Institutes of Health grants GM54995 (to J.R.H.) and Center grant ES06676.

ABBREVIATIONS: MDZ, midazolam; P450, cytochrome P450; SRS, substrate recognition site; ANF, α -naphthoflavone; AFB1, aflatoxin B₁; RPR 106541, 20R-16 α ,17 α -[butylidenebis(oxy)]-6 α ,9 α -difluoro-11 β -hydroxy-17 β -(methylthio)androsta-4-en-3-one; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonic acid; DOPC, dioleoylphosphatidylcholine; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography.

much higher 1'-OH/4-OH ratio of MDZ metabolism than CYP3A4 (Gorski et al., 1994; Kuehl et al., 2001).

The oxidation of MDZ by CYP3A4 has been the focus of many in vitro investigations (Kronbach et al., 1989; Gorski et al., 1994; Ghosal et al., 1996; Maenpaa et al., 1998; Hosea et al., 2000; Wang et al., 2000). These studies have found very different K_M values for the formation of 1'-OH MDZ compared with 4-OH MDZ by CYP3A4. Production of the two metabolites has also been reported to be stimulated/inhibited differentially by various compounds. Thus, whereas the presence of ANF stimulated 1'-OH MDZ formation, 4-OH MDZ formation was decreased or unaltered. In contrast, testosterone increased 4-OH MDZ and decreased 1'-OH MDZ formation. A recent study by Hosea et al. (2000) also reported two distinct K_i values for inhibition of 1'-OH and 4-OH MDZ formation by a peptide (YPFP-NH₂) shown to interact with CYP3A4. These and other studies have led to the suggestion that MDZ binds at two different locations in the CYP3A4 active site (Ghosal et al., 1996; Hosea et al., 2000).

In recent years, understanding structure-function relationships of CYP3A4 has been a major focus of our laboratory (see Domanski and Halpert 2001 and references therein). A sequence alignment with bacterial P450s of known structure was used to localize putative SRSs (Gotoh, 1992) within CYP3A4 (Szkwarz and Halpert, 1997) based on a similar successful approach with P450 family 2 enzymes. Site-directed mutagenesis, homology modeling, and functional analysis using substrates such as progesterone, testosterone, AFB1, RPR 106541, 7-alkoxycoumarins, ANF, and 7-benzoyloxy-4-trifluoromethylcoumarin led to the identification of a number of SRS residues that are involved in determining substrate specificity. These studies have also provided strong evidence in support of the hypothesis that there are multiple substrate binding sites in CYP3A4 (Shou et al., 1994; Korzekwa et al., 1998). In the present study, we have investigated MDZ metabolism by CYP3A4 wild-type and 30 mutants generated at 15 different SRS positions to systematically evaluate the role of various SRS residues in substrate oxidation and the possible existence of multiple MDZ binding sites. The study showed that MDZ metabolism causes CYP3A4 inactivation and suggests that such enzyme inactivation is related to the 1'-OH MDZ metabolic pathway. The SRS residues at which amino acid substitution showed the most significant effect on regioselectivity of MDZ metabolism were Phe-108, Ser-119, Ile-120, Leu-210, Ile-301, Phe-304, Ala-305, Tyr-307, Thr-309, Leu-373, and Leu-479. Correlational analysis of 1'-OH formation versus 4-OH

formation and analysis of the effect of side chain size on the metabolite profile by all mutants, along with steady-state kinetic analyses and substrate binding studies of selected mutants suggest that the two putative MDZ binding sites in CYP3A4 are near each other.

Experimental Procedures.

Materials. MDZ, flunitrazepam, NADPH, CHAPS, and DOPC were purchased from Sigma Chemical Co. (St. Louis, MO). HEPES was obtained from CalBiochem Corp. (La Jolla, CA). The Expand PCR Kit and Rapid Ligation kits were obtained from Roche (Indianapolis, IN). The QuickChange site-directed mutagenesis kit and GeneClean kit were from Stratagene (La Jolla, CA) and BIO 101 (Carlsbad, CA), respectively. The bovine serum albumin protein assay kit was purchased from Pierce (Rockford, IL). Thin-layer chromatography plates [silica gel, 250 μ m; Si 250F (C19)] were purchased from J. T. Baker, Inc. (Phillipsburg, NJ). 1'-OH and 4-OH MDZ were gifts from Dr J. C. Stevens (Pharmacia, Kalamazoo, MI). Recombinant NADPH-cytochrome P450 reductase and cytochrome *b₅* from rat liver were prepared as described earlier (Harlow and Halpert, 1997). All other chemicals were of the highest grade available and were obtained from standard commercial sources.

Mutant Construction. Mutants P107A, P107W, and F108A were generated with the megaprimer method. In the first amplification reaction, the mutagenic primer (see Fig. 1) and a pSE380 3'-specific primer were used with pSE3A4His as the template (Domanski et al., 1998). The product was used in a second amplification as a primer in conjunction with a pSE380 5'-specific primer and pSE3A4His again as the template. To increase the amount of product for further cloning, the product of the second PCR was used in a third reaction as the template with the same primers used in round 2. Both pSE3A4His and the final products were digested with *Nco*I and *Bam*HI, the desired bands were purified with GeneClean (Bio101, Vista, CA), ligated together, and transformed into *E. coli* DH5 α cells. Because the P107A, P107W, and F108A mutagenic primers contained alterations that removed a *Stu*I site, clones were screened for the absence of this site.

Mutant I120A was generated by the overlap PCR extension method. Plasmid pSE3A4His was used as a template. In two separate reactions, the mutagenic forward primer (Fig. 1) and downstream vector primer or the mutagenic reverse primer and upstream vector primer were used. Subsequently, the PCR-amplified fragments served as the template to amplify the full-length coding region with two vector primers. The full-length mutated fragment was purified with the GeneClean Kit and ligated into pSE3A4His that was digested with the same restriction enzymes (*Aat*II and *Nco*I) and purified. Because the mutagenic primers contained alterations that created a *Pst*I site, clones were screened for the presence of this site.

Mutant Y307A was generated using the Expand PCR kit as described previously (Domanski et al., 1998) using pSE3A4His as the

P107A	5' GTC TTC ACA AAC CGG AGG <u>GCT</u> TTT GGT CCA GTG GGA
P107W	5' GTC TTC ACA AAC CGG AGG <u>TGG</u> TTT GGT CCA GTG GGA
F108A	5' GTC TTC ACA AAC CGG AGG <u>CCC</u> <u>GCT</u> GGT CCA GTG GGATT
I120A (forward)	5' GT GCC ATC TCT <u>GCA</u> GCT GAG G
I120A (reverse)	5' CTT CAT CCT CAG CTG CAG A
Y307A (forward)	5' ATC TTT ATT TTT GCT GGC <u>GCT</u> GAA ACC ACG AGC
Y307A (reverse)	5' AGC AAA AAT AAA GAT AAT TGA TTG
L479A (forward)	5' C CTG AAA TTA AGC <u>GCT</u> GGA GGA CTT C
L479A (reverse)	5' GAA GTC CTC CAG CGC TTA ATT TCA G

Fig. 1. Primers used for generation of CYP3A4 cDNA mutants. The mutated nucleotides are underlined. The generations of unique restriction sites due to these mutations are represented in bold (*Pst*I for I120A and *Eco*47III for L479A). The deletion of a *Stu*I site due to the mutations (in the case of P107A, P107W, and F108A) is represented in italics.

template and Y307A forward and reverse primers (Fig. 1). The mutation was verified by sequencing. Mutant L479A was generated using the QuickChange site-directed mutagenesis kit using pSE3A4His as the template. Because the mutagenic primers contained alterations that created an *Eco47III* site, clones were screened for the presence of this site. In all cases, the entire coding region was sequenced to verify the presence of the desired mutation and to ensure that no extraneous changes were present.

Expression and Purification of CYP3A4 and Mutants. Wild-type CYP3A4, as well as newly and previously generated mutants, were expressed as His-tagged proteins in *Escherichia coli* TOPP3 (or DH5 α) cells and purified using Talon metal affinity resin (CLONTECH, Palo Alto, CA) as described earlier (Harlow and Halpert, 1997, 1998; He et al., 1997; Domanski et al., 1998, 2000, 2001; Wang et al., 1998; Stevens et al., 1999; Khan and Halpert, 2000; Roussel et al., 2000). The P450 content was determined by carbon monoxide difference spectra in the presence of 1% Triton X-100 added to the protein sample before dilution with microsome solubilization buffer containing 100 mM potassium phosphate, pH 7.3, 20% glycerol, 0.5% sodium cholate, 0.4% Renex, and 1.0 mM EDTA. Total protein content in each sample was determined with the bicinchoninic acid protein assay kit.

MDZ Hydroxylase Assay. The reconstituted system for the assay contained 10 pmol of purified P450, 20 pmol of rat liver cytochrome *b*₅, 40 pmol of recombinant NADPH-cytochrome P450 reductase, 0.04% CHAPS, and 1 mg/ml of DOPC (Harlow and Halpert, 1997). The mixture was preincubated for 10 min at room temperature. MDZ dissolved in methanol was added to the reconstituted protein system in 50 mM HEPES buffer, pH 7.6, and 15 mM MgCl₂. The protein-substrate mixture was further incubated for 5 min at 37°C, and the reaction was initiated by adding NADPH (1 mM final concentration). The total reaction volume of the assay was 100 μ l. After 5 min of incubation at 37°C, the reactions were stopped by addition of 100 μ l of methanol containing 5 nmol of flunitrazepam as an internal standard. For all mutants as well as wild-type, the assay was carried out at least twice to confirm the validity of the metabolic profiles.

The metabolite extraction as well as HPLC analysis was essentially carried out according to the method developed by Gorski et al. (1994). The metabolites were extracted twice with 2.5 ml of cyclohexane: methylene chloride (7: 3) after addition of 250 μ l of 0.2 mM sodium borate (pH 9.6). The extracted metabolites were dried under nitrogen, and the residue was resuspended in 200 μ l of the mobile phase [methanol: phosphate buffer (pH 7.4): tetrahydrofuran, 52: 46: 2]. Fifty- μ l of the mixture was loaded for HPLC chromatographic analysis.

HPLC Analysis. The HPLC system consisted of two Beckman 110 solvent delivery modules, a Beckman 421A system controller (Beckman, Berkeley, CA), a 50- μ l injection loop, a spectroflow 757 UV-absorbance detector (Kratos Analytical, Ramsey, NJ), and a Spectra-Physics SP4270 Integrator (Spectra-Physics, Piscataway, NJ). An ultrasphere ODS column (5 μ m $\times$ 250 mm $\times$ 4.6 mm; Beckman Coulter, Fullerton, CA) was used with an ultrasphere C18 guard column (5 μ m $\times$ 7.5 mm $\times$ 4.6 mm; Alltech, Deerfield, IL). The separation of the metabolites of MDZ was achieved isocratically using the mobile phase (methanol/phosphate buffer, pH 7.4/ tetrahydrofuran, 52:46:2). The flow rate was 1.0 ml/min and the UV detector was set at 230 nm. All chromatographic separations were performed at room temperature.

Inactivation Assays. The reconstitution conditions were essentially the same as described above. The preincubation mixture contained 35 pmol of purified P450, 70 pmol of rat liver cytochrome *b*₅, 140 pmol of recombinant NADPH-cytochrome P450 reductase, 0.04% CHAPS, and 1 mg/ml of DOPC. After incubating for 10 min at room temperature, MDZ was added to the reconstituted protein system in 50 mM HEPES buffer, pH 7.6, and 15 mM MgCl₂. The protein-substrate mixture was further incubated for 5 min at 37°C, and the reaction was initiated by adding NADPH (1 mM final concentration). The total volume of the mixture was 560 μ l. Aliquots (80 μ l) of this

mixture were taken at various time intervals (0–6 min) and added to a secondary reaction mixture (20 μ l) for determination of the residual progesterone hydroxylase activity ([progesterone] = 25 μ M). The reaction was stopped after 6 min by the addition of 50 μ l of tetrahydrofuran, and 50 μ l of the reaction mixture was spotted on the preadsorbent loading zone of a thin-layer chromatography plate (Baker silica gel; 250 ml, Si 250F). The plate was developed twice in benzene/ethyl acetate/acetone [10:1:1 (v/v/v)]. Metabolites were visualized by autoradiography and identified by comparison with unlabeled standards. The radioactive areas from the plate were scraped into scintillation vials, and the metabolites were quantified by liquid scintillation counting.

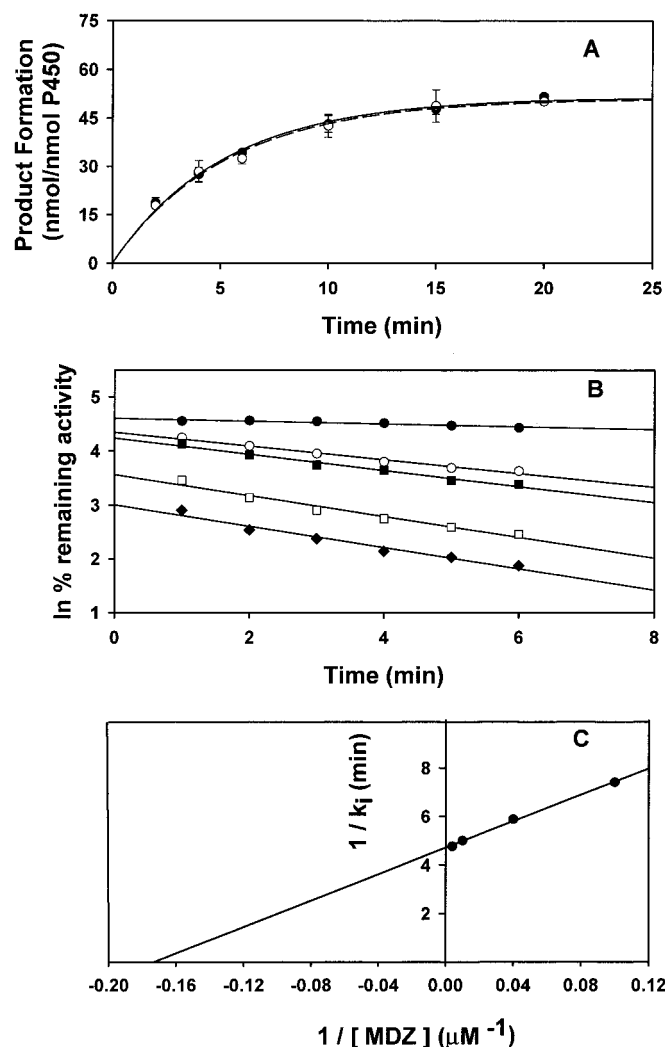


Fig. 2. A, the time dependence of MDZ (250 μ M) hydroxylation by CYP3A4. The details of the reaction are described under *Experimental Procedures*. ● and ○ represent 4-OH and 1'-OH MDZ, respectively. The solid lines through the experimental data points show the fit to the first-order exponential rate equation, $r = R_{\max} \times \exp(-k_{\text{obs}} \times t)$ (see *Results* for detail). B, time- and concentration-dependent inactivation of the wild-type enzyme by MDZ as measured by the percentage decrease in progesterone 6 β -hydroxylation. The details of the inactivation reactions are described under *Experimental Procedures*. The concentrations of MDZ were 0 (●), 10 (○), 25 (■), 100 (□), and 250 (◆) μ M. The lines shown through experimental points were generated by linear regression analysis of the natural logarithm of the residual activity as a function of time. Rate constants of inactivation (k_i) are derived from the negative slope of the lines, whereas the extent of reversible inhibition is reflected by a decrease in the extrapolated activity at zero preincubation time compared with the methanol control. C, double-reciprocal plot of the rate constants of inactivation as a function of MDZ concentration.

Spectral Binding Studies. Binding spectra were recorded on a Shimadzu-2600 spectrophotometer fitted with a temperature controller (TCC-240A). A solution (0.8 ml) containing 0.5 μM protein in 100 mM phosphate, pH 7.4, was divided into two quartz cuvettes (10-mm path length) and a baseline was recorded between 350 and 500 nm. An aliquot of substrate in methanol was then added to the sample cuvette, and the same amount of methanol was added to the reference cuvette. The difference spectra were obtained after the system reached equilibrium (3 min). All the spectra were recorded at 37°C.

Results

Inactivation of CYP3A4 by MDZ. The oxidation of MDZ by CYP3A4 generated two hydroxylated products, 1'-OH MDZ and 4-OH MDZ, as reported earlier (Gorski et al., 1994). The reaction was found to be nonlinear with respect to time (Fig. 2A), suggesting that MDZ might be inactivating the enzyme, as reported previously (Podoll et al., 1995; Schrag and Wieners, 2001). The nonlinear behavior of MDZ metabolism by CYP3A4 could be fitted to a first-order exponential rate equation, $r = R_{\max} \times \exp(-k_{\text{obs}} \times t)$, where k_{obs} represents the rate constant, and R and $R_{\max}$ are the nanomoles of product formed per nanomole P450 at a particular time (t) and at infinity, respectively. At 250 μM MDZ, the values of $R_{\max}$ and k_{obs} for 4-OH MDZ were 51.4 ± 1.4 nmol/nmol P450 and 0.19 ± 0.01 min⁻¹, respectively, and for 1'-OH MDZ, they were 51.0 ± 1.5 nmol/nmol P450 and 0.19 ± 0.02 min⁻¹, respectively. If the decrease in the rate of metabolism of MDZ over time is caused by the inactivation of the enzyme, the k_{obs} determined by this fit should be equal to the rate constant of inactivation at the same MDZ concentration. To further investigate this possibility, the kinetics of CYP3A4 inactivation by MDZ was studied by measuring the time-dependent decrease in progesterone 6 β -hydroxylation. The enzyme was inactivated by MDZ in a time- and concentration-dependent manner (Fig. 2B). The inactivation followed pseudo-first-order kinetics (Fig. 2B), and was saturable with MDZ (Fig. 2C). The kinetic constants were determined from the fit of the rate constants of inactivation versus MDZ concentration to the Michaelis-Menten equation. The concentration of inactivator required for half-maximal inactivation (K_I) was 5.8 ± 0.3 μM , and the maximal rate constant of inactivation

at saturation (k_{inact}) was 0.15 ± 0.00 min⁻¹. Thus, the k_{inact} determined above showed good agreement with the k_{obs} obtained from the fit of the time dependence of MDZ metabolism by the enzyme at saturating substrate (see next paragraph).

MDZ Metabolism by Wild-Type Enzyme. Despite the inactivation of CYP3A4 by MDZ, analysis of the protein concentration dependence of MDZ metabolism (5-min incubation) showed that the reaction was linear between 25 and 125 nM P450 (2.5–12.5 pmol in 100- μl reaction volume; data not shown). Therefore, all subsequent reactions were carried out using 100 nM enzyme and a 5-min incubation time unless stated otherwise. Under these conditions, analysis of steady-state kinetics of MDZ oxidation by the wild-type enzyme gave a hyperbolic V versus S plot for both products (data not shown). Kinetic parameters determined from the fit to the Michaelis-Menten equation showed two very distinct K_M values (3.7 ± 0.9 and 64 ± 4 μM for 1'-OH and 4-OH MDZ, respectively; Table 1), as reported by others (Gorski et al., 1994; Ghosal et al., 1996; Hosea et al., 2000).

Effect of SRS Substitutions. Recent studies from our laboratory involving the use of site-directed mutagenesis based on structure-based sequence alignments have proven informative in understanding the structure-function relationships of CYP3A4 (Harlow and Halpert, 1997, 1998; He et al., 1997; Domanski et al., 1998, 2000, 2001; Wang et al., 1998; Stevens et al., 1999; Khan and Halpert, 2000; Roussel et al., 2000; Xue et al., 2001). These mutagenesis studies were the first to establish that Gotoh's SRS model (Gotoh, 1992) is also applicable to the CYP3A subfamily and have identified counterparts to all the active-site residues inferred from the X-ray crystal structure of rabbit CYP2C5 (Williams et al., 2000; reviewed in Domanski and Halpert, 2001). Here, we systematically evaluated SRS residues with the goals of elucidating the role of these residues in MDZ regioselectivity and of mapping the two proposed MDZ binding sites (Ghosal et al., 1996; Hosea et al., 2000). For this purpose, 30 mutants generated at 15 different SRS positions, which outline almost the entire putative CYP3A4 active site, were analyzed for MDZ oxidation. All 15 SRS residues selected for the study

TABLE 1

Kinetic constants determined from the concentration dependence of MDZ oxidation by CYP3A4 wild-type and mutants.

The values in parentheses show the average deviation obtained from the fit of the Michaelis-Menten equation to a single kinetic data set. The experiments were done multiple times ($n = 2-4$) using multiple protein preparations without significant change in the ratio of $V_{\max}/K_M(1'\text{-OH})/V_{\max}/K_M(4\text{-OH})$.

	1'-OH			4-OH			Ratio
	$V_{\max}$	K_M	$V_{\max}/K_M$	$V_{\max}$	K_M	$V_{\max}/K_M$	
	min ⁻¹	μM	$\mu\text{M}/\text{min}$	min ⁻¹	μM	$\mu\text{M}/\text{min}$	$V_{\max}/K_M(1'\text{-OH})/V_{\max}/K_M(4\text{-OH})$
WT	8.9 (0.3)	3.7 (0.9)	2.4	10.9 (0.3)	64.0 (4.0)	0.17	14
S119A ^a	12.7 (0.6)	27.3 (4.5)	0.46	40.0 (2.2)	114.1 (12.8)	0.35	1.3
I120W ^{b,c}	23.5 (0.7)	1.9 (0.6)	12.1	8.5 (0.3)	93.7 (8.0)	0.09	134
L210A ^a	20.5 (0.9)	22.3 (3.4)	0.92	28.6 (0.7)	42.0 (3.1)	0.68	1.4
F304A ^a	12.6 (0.6)	12.9 (2.6)	0.98	23.9 (1.3)	65.3 (8.8)	0.37	2.7
F304W ^{b,c}	17.5 (0.4)	2.4 (0.5)	7.2	6.9 (1.0)	87.8 (28.3)	0.08	92
T309F	2.7 (0.1)	3.3 (0.9)	0.83				—
I369W				4.1 (0.4)	89.0 (21.3)	0.05	—
L373F ^d	5.8 (0.3)	36.4 (6.6)	0.16	17.3 (0.7)	154.6 (12.1)	0.11	1.4
L479F ^a	16.2 (0.7)	9.3 (2.1)	1.75	7.0 (0.9)	83.9 (23.6)	0.08	21

^{a,b} Due to very high rate of metabolite formation with some of the mutants, the incubations were done using 5 (a) and 3 (b) pmol of the enzyme in these cases.

^c The reaction kinetics of 1'-OH MDZ formation by I120W and F304W possibly showed a small extent of inhibition at higher substrate concentrations (see Fig. 4) as reported for wild-type CYP3A4 (Gorski et al., 1994; Ghosal et al., 1996; Perloff et al., 2000). A reanalysis of the reaction kinetics by a model incorporating Michaelis-Menton kinetics with uncompetitive substrate inhibition showed no significant change in the K_M and $V_{\max}$ values (shown by dotted lines in Fig. 4) compared with those determined using the Michaelis-Menton equation. The K_{inb} determined from these fits showed very large standard deviations and is therefore not reported.

^d Eiselt et al. (2001).

have been either previously shown to play an important role in the oxidation of one or more CYP3A4 substrates (Doman-ski and Halpert, 2001) or were selected based on analogy with the active-sites in the CYP2C5 or P450eryF crystal structures (Cupp-Vickery et al., 2000; Williams et al., 2000). Furthermore, unlike some of the other P450s, CYP3A4 does not show any special charge requirement in the active site for the various substrates it metabolizes [see Khan and Halpert (2000) and references therein]. Therefore, to maximize the effect of side chain substitutions, for each selected SRS position one larger and one smaller residue was chosen. Because of the large difference in the two K_M values for MDZ metabolism by CYP3A4 and the ensuing concentration dependence of the metabolite profile, metabolism of MDZ by the mutants was studied at two different concentrations, 25 and 250 μM (Fig. 3). MDZ metabolism by CYP3A4 wild-type showed a decrease of more than 3-fold in the ratio of 1'-OH MDZ to 4-OH MDZ between the two different concentrations.

SRS-1 encompasses the B' helix, which is one of the most variably positioned regular secondary-structure elements among P450s of known crystal structure, with very different lengths and orientations as well as very low sequence identity (Hasemann et al., 1995; Szklarz and Halpert, 1997). Four

residues, Pro-107, Phe-108, Ser-119, and Ile-120, were explored from SRS-1 (Fig. 3). Ser-119 has been recently identified as the residue whose substitution has the most drastic effect on regioselectivity of steroid hydroxylation by CYP3A4, changing its preference from testosterone 6 β - to 2 β -hydroxylation (Roussel et al., 2000). Pro-107, Phe-108, and Ile-120 were selected on the basis of sequence alignment and analogy with P450eryF and CYP2C5 (Cupp-Vickery et al., 2000; Williams et al., 2000). Among the SRS-1 mutants tested, F108W, S119A, and I120W showed the most significant change in activity (Fig. 3). Both F108W and I120W exhibited higher preference¹ for 1'-hydroxylation compared with the wild-type enzyme. In contrast, S119A showed much higher preference for 4-hydroxylation and exhibited the highest total MDZ hydroxylation activity among all mutants tested. Analysis of the substrate dependence of MDZ hydroxylation by S119A showed an almost 4-fold increase in the V_{max} for 4-OH MDZ formation and a 7- to 8-fold increase in the K_M for 1'-OH MDZ formation (Fig. 4 and Table 1). This leads to a more

¹ The terms "preference for" or "preferential increase or decrease" are used to denote an altered metabolite profile in assays performed at 25 and/or 250 μM MDZ.

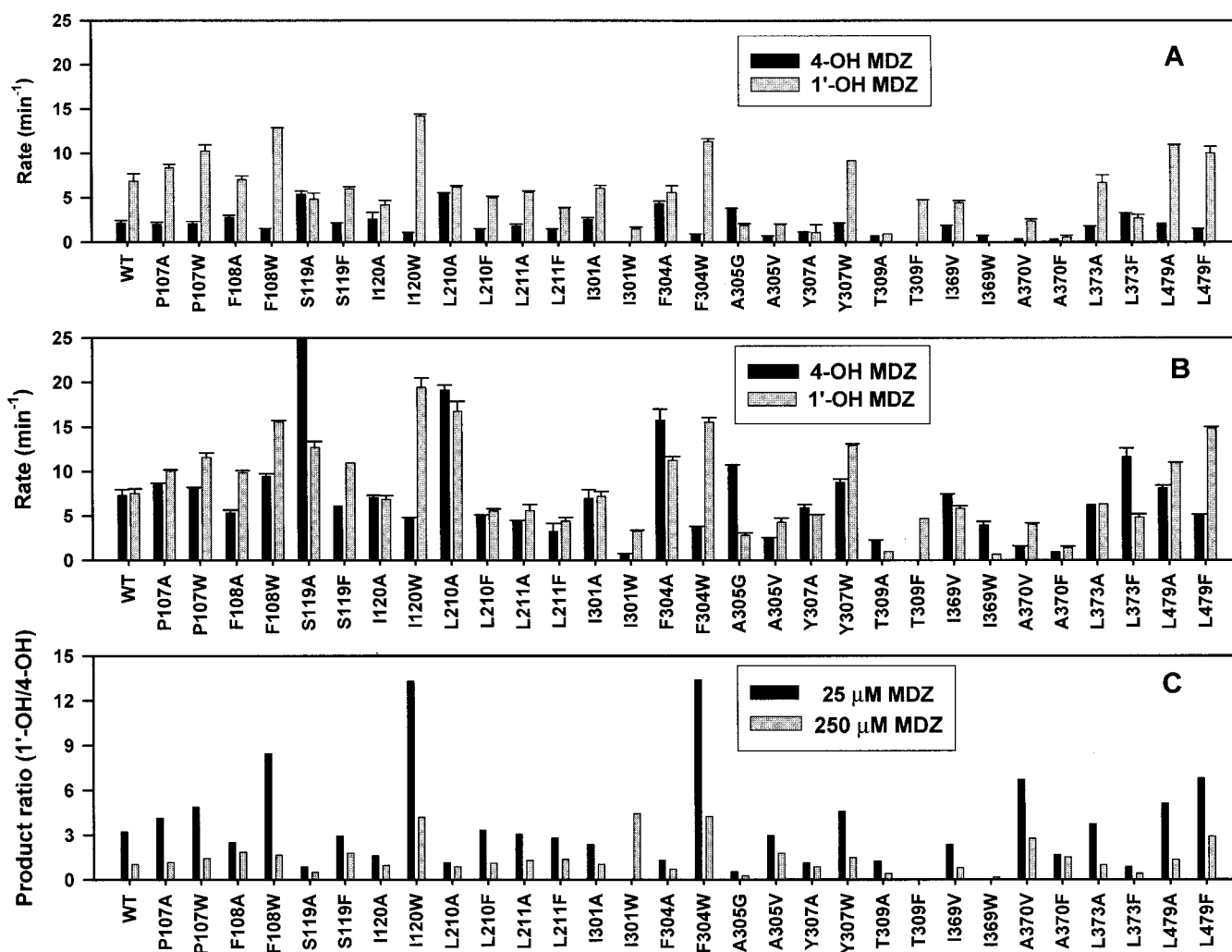


Fig. 3. The metabolite profiles of the wild-type and various CYP3A4 SRS mutants at 25 μM (A) and 250 μM (B) MDZ. Assay conditions are described under *Experimental Procedures*. The error bars represent average deviation from duplicate incubations. C, the ratio of 1'-OH/4-OH product formation at both concentrations.

than 10-fold decrease in selectivity² for 1'-OH versus 4-OH MDZ compared with the wild-type (reflected in the change of the ratio of $V_{\max}/K_M(1'\text{-OH})/V_{\max}/K_M(4\text{-OH})$). Similar analysis of I120W showed that the 10-fold increase in selectivity for 1'-OH versus 4-OH MDZ compared with the wild-type is caused mainly by an increase of nearly 3-fold in the $V_{\max}$ and a 2-fold decrease in the K_M for 1'-OH MDZ formation. There is also a 2-fold decrease in the $V_{\max}/K_M$ for 4-OH MDZ by the mutant compared with the wild-type (Fig. 4 and Table 1).

SRS-2, which encompasses the F and G helices, is another region that varies considerably both in length and sequence (Hasemann et al., 1995; Szklarz and Halpert, 1997). L210A was the only mutant among the four from SRS-2 to show a significant change in MDZ oxidation (Fig. 3). This mutation has also been reported to have a significant effect on the ability of the enzyme to metabolize compounds such as AFB1, RPR 106541, and 7-hexoxycoumarin (Wang et al., 1998; Stevens et al., 1999; Khan and Halpert, 2000). Kinetic analysis showed a 2- to 3-fold increase in the $V_{\max}$ for both products, and a 6-fold increase in the K_M for 1'-OH formation, leading to a 10-fold decrease in the selectivity for 1'-OH versus 4-OH MDZ formation by the mutant compared with the wild-type (Table 1).

² Selectivity refers to catalytic efficiency ($V_{\max}/K_M$) for 1'-OH MDZ formation compared with 4-OH MDZ formation.

SRS-4 is located in the I helix and is one of the most conserved regions in P450s. Several I helix residues have been shown to be involved in proton delivery as well as substrate specificity and/or reaction kinetics in various P450 enzymes (Hasemann et al., 1995; Szklarz and Halpert, 1997). Substitutions at all five sites explored caused significant changes in the MDZ product profile (Fig. 3), confirming the importance of SRS-4 in substrate metabolism and selectivity. I301W, although exhibiting lower activity compared with wild-type, showed a preference for 1'-OH hydroxylation. Phe-304, which has been shown to play a crucial role in substrate metabolism and cooperativity (Harlow and Halpert, 1998; Domanski et al., 1998; 2000) also showed profound involvement in the MDZ metabolite profile. Substitution of this residue with alanine increased the preference for 4-OH MDZ formation, whereas a tryptophan substitution caused the opposite effect. Kinetic analysis of F304A showed a nearly 4-fold increase in the K_M for 1'-OH MDZ formation and more than a 2-fold increase in the $V_{\max}$ for 4-OH MDZ formation (Fig. 4 and Table 1). This leads to a decrease of more than 5-fold in selectivity for 1'-OH versus 4-OH MDZ compared with the wild-type. In contrast, similar analysis of F304W showed that the change in function is caused mainly by a 2-fold increase in the $V_{\max}$ for 1'-OH MDZ formation and small decreases in the $V_{\max}$ for 4-OH MDZ and the K_M for

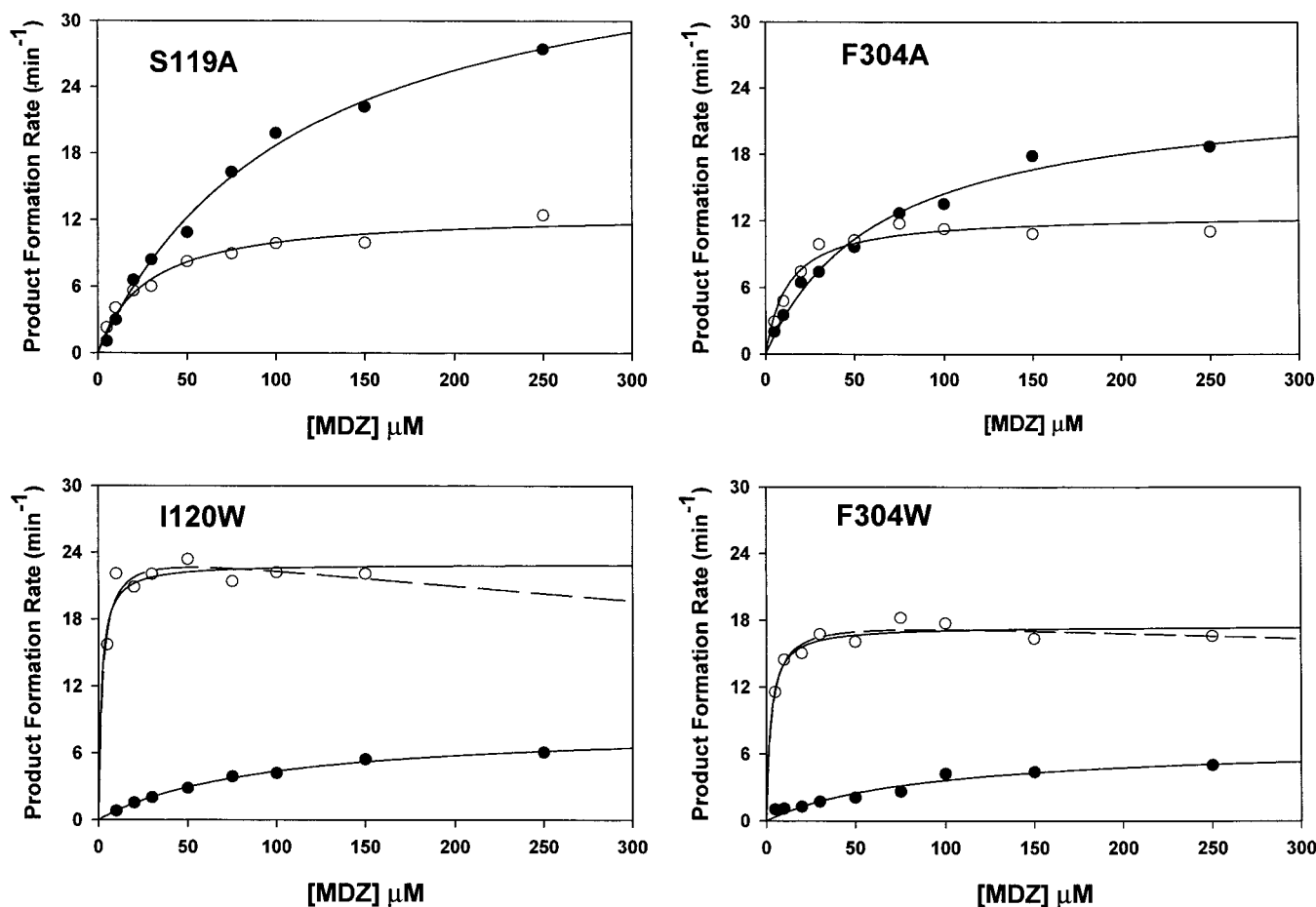


Fig. 4. Midazolam hydroxylation kinetics by S119A, I120W, F304A, and F304W. ● and ○ represent 4-OH and 1'-OH MDZ, respectively. The solid lines through experimental data points show the fit to the Michaelis-Menten equation, and the dotted lines through the I120W and F304W data points show the fit to a modified Michaelis-Menten kinetics with uncompetitive substrate inhibition (Perloff et al., 2000).

TABLE 2

Relative MDZ metabolite formation by mutants compared with wild-type CYP3A4

The table provides the comparison at 25 μ M MDZ. For each SRS residue, one smaller and one larger substitution was considered. The wild-type CYP3A4 MDZ hydroxylation activity (6.9 and 2.2 nmol/min/nmol of P450 for 1'-OH and 4-OH MDZ, respectively) was considered 100% for each product. The relative ratio for the wild type is therefore 1.0, which is different from the actual metabolite ratio of 3.1.

	1'-OH	4-OH	1'-OH/4-OH		1'-OH	4-OH	1'-OH/4-OH
WT	100	100	1.0	WT	100	100	1.0
P107A	121	93	1.3	P107W	149	98	1.5
F108A	102	130	0.8	F108W	187	70	2.7
S119A	70	250	0.3	S119F	87	95	0.9
I120A	61	120	0.5	I120W	206	49	4.2
L210A	90	251	0.4	L210F	72	70	1.0
L211A	81	84	1.0	L211F	56	64	0.9
I301A	88	119	0.7	I301W	22	0	
F304A	81	201	0.4	F304W	164	39	4.2
A305G	28	172	0.2	A305V	29	31	1.0
Y307A	16	45	0.4	Y307W	133	93	1.4
T309A	13	33	0.4	T309F	69	0	
I369V	64	87	0.7	I369W	0	30	0.0
A370V	35	17	2.1	A370F	8	16	0.5
L373A	97	83	1.2	L373F	39	143	0.3
L479A	159	99	1.6	L479F	146	68	2.2

1'-OH MDZ (Fig. 4 and Table 1). Overall, this leads to an increase of more than 7-fold in the selectivity for 1'-OH versus 4-OH MDZ compared with the wild-type. Mutant A305G showed a preference for 4-hydroxylation, whereas A305V demonstrated a much lower activity, but a preference for 1'-hydroxylation (Fig. 3). Y307A showed a much lower rate of 1'-OH hydroxylation at 25 μ M MDZ, although its activity was similar to the wild-type at 250 μ M (Fig. 3). In contrast, Y307W displayed slightly higher preference for 1'-hydroxylation at both concentrations compared with the wild-type (Fig. 3). Thr-309, and the corresponding residue in various P450s, has been extensively studied because of its proposed involvement in proton delivery to the substrate during the P450 catalytic cycle. As reported previously, substitution of Thr-309 by alanine did not have a significant effect on the testosterone or progesterone hydroxylase activity of the enzyme (Domanski et al., 1998). With MDZ, T309A showed one tenth of the wild-type hydroxylation activity (Fig. 3). Interestingly, although T309F showed lower activity, it exclusively hydroxylated MDZ to the 1'-OH product at all concentrations (Fig. 3 and Table 1). As we reported recently, T309F does not have any significant activity toward testosterone, progesterone, or ANF, but has slightly higher than wild-type activity with 7-benzoyloxy-4-trifluoromethylcoumarin (Domanski et al., 2001). Kinetic analysis showed a decrease in the $V_{\max}$ value for 1'-OH MDZ formation, whereas the K_M value remained unchanged, leading to a 3-fold decrease in the catalytic efficiency of 1'-OH MDZ formation compared with the wild-type (Table 1).

SRS-5, located in regions containing β 6-1 and β 1-4, is among the most conserved regions, which is reflected in its role in heme binding (Hasemann et al., 1995; Szklarz and Halpert, 1997). Three residues from SRS-5, Ile-369, Ala-370, and Leu-373, were examined (Fig. 3). Of all the mutants in this study, I369W displayed the highest preference for 4-OH MDZ formation. The $V_{\max}$ value for 4-OH MDZ formation by this mutant was found to decrease approximately 2-fold compared with wild-type, with little change in the K_M value, leading to a 3-fold decrease in the catalytic efficiency of 4-OH MDZ formation compared with the wild-type (Table 1). The kinetic parameters for 1'-OH MDZ could not be determined because of the very low rate of formation. The only other

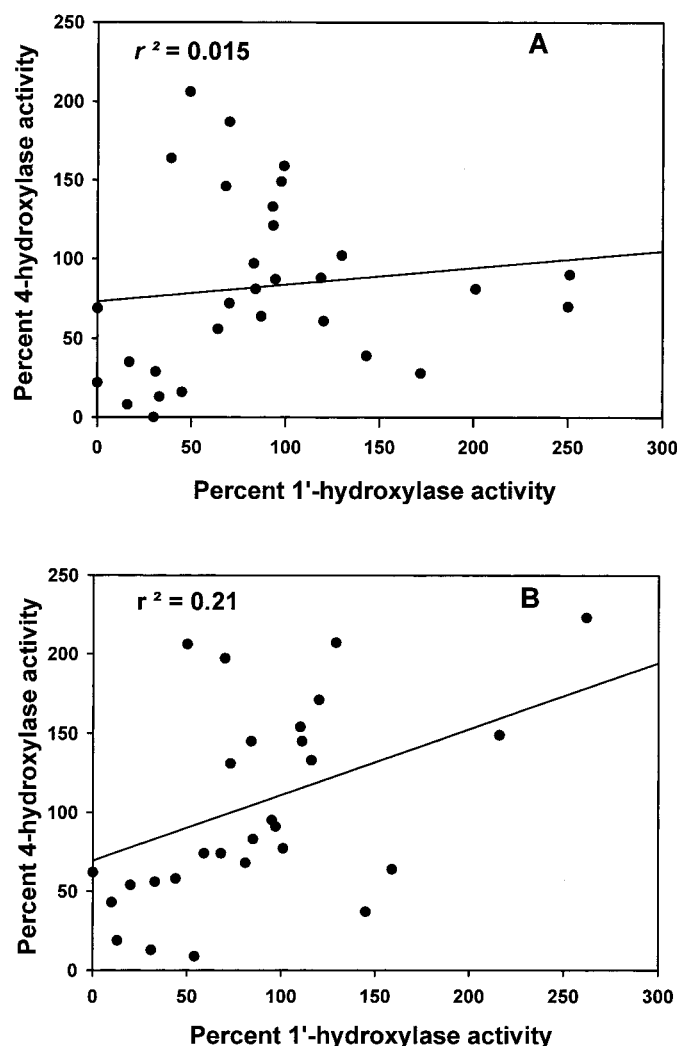


Fig. 5. A plot showing the correlational analysis of 4-OH formation versus 1'-OH formation at 25 μ M (A) and 250 μ M (B) MDZ. The r^2 values were determined from linear regression of all 30 mutants.

SRS-5 mutant to show a significant change in MDZ hydroxylation was L373F, which is an allelic variant recently reported in humans (Eiselt et al., 2001). As shown in Table 1, L373F exhibits a 10-fold decrease in the selectivity for 1'-OH versus 4-OH MDZ compared with the wild-type.

The only residue explored from SRS-6 was Leu-479 (Fig. 3). Substitution of this residue with either alanine or phenylalanine increased the ability of CYP3A4 to oxidize MDZ. Both mutants exhibited a slightly increased preference for 1'-OH MDZ. The kinetic analysis of L479F indicates complex behavior. Whereas the K_M for both products and the V_{max} for 1'-OH MDZ showed increases, the V_{max} for 4-OH MDZ decreased slightly, the net result being a 1.5-fold increase in the selectivity for 1'-OH versus 4-OH MDZ compared with the wild-type (Table 1).

In summary, several SRS residues seem to have a very significant effect on MDZ metabolism. Some of the most influential residues among these are Phe-108, Ser-119, Ile-120, Leu-210, Ile-301, Phe-304, Ala-305, Tyr-307, Thr-309, Ile-369, Leu-373, and Leu-479. Interestingly, among the 15

residues tested in this study, residues Pro-107, Phe-108, Ile-120, Leu-210, Ile-369, and Leu-479 correspond to 6 of the 17 divergent amino acids within the six putative SRS of 3A4 and 3A5 (Wang et al., 1998; Xue et al., 2001). The substitution of two of these residues, Leu-210 and Ile-369, with the Phe from CYP3A5 caused no significant change in the regioselectivity of MDZ oxidation. Mutants F108W, I120W, and L479A/W all showed a higher metabolite ratio of 1'-OH/4-OH MDZ compared with the wild-type CYP3A4, similar to what is observed for the wild-type CYP3A5. However, it is important to recognize that these changes do not correspond to the actual CYP3A5 residues.

A comparison of 4-OH and 1'-OH MDZ formation at 25 μM MDZ by the mutants expressed as the percentage of wild-type activity showed another very interesting trend.³ As shown in Table 2, the substitution of residues Ser-119, Ile-120, Leu-210, Phe-304, Ala-305, Tyr-307, and Thr-309 with a

³ A comparison of 4-OH and 1'-OH MDZ formation at 250 μM MDZ by the mutants also showed a very similar trend.

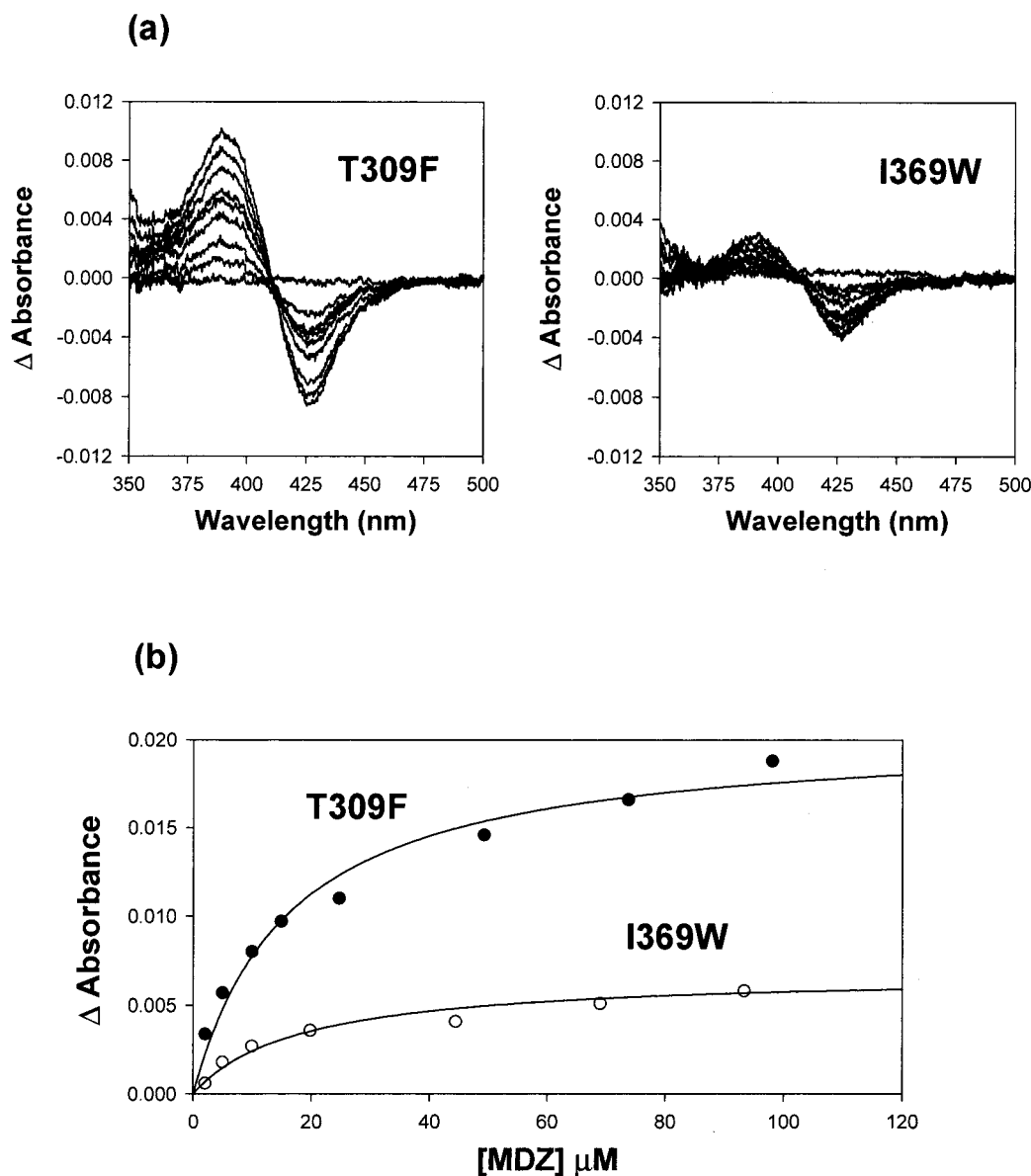


Fig. 6. Spectral titration of T309F and I369W with increasing concentrations of MDZ (a) and the plots of change in absorbance versus [MDZ] (b). The solid lines through the experimental data points show the fit to the equation $\Delta A = \Delta A_{max}S / (K_D + S)$, where K_D is the dissociation constant, and ΔA and ΔA_{max} are the change in absorbance at a particular substrate concentration (S) and at a saturating substrate concentration, respectively. The MDZ concentration as well as absorbance change have been adjusted for the dilution caused by the addition of MDZ. The protein concentration was 0.5 μM in both cases.

TABLE 3

Midazolam dissociation constant, maximal absorbance change and percent spin-state change on enzyme-substrate complex formation for the wild-type CYP3A4 and selected mutants. An extinction coefficient of $126 \text{ mM}^{-1} \text{ cm}^{-1}$ has been used to calculate the percentage spin change due to MDZ binding with the enzyme (Harlow and Halpert, 1998). The values in parentheses show the average deviation obtained from the fit of the equation $\Delta A = \Delta A_{\text{max}} S / (K_D + S)$, where K_D is the dissociation constant and ΔA and ΔA_{max} are the change in absorbance at a particular substrate concentration (S) and at saturating substrate concentration, respectively.

	Trough	Peak	ΔA_{max}	K_D	Spin Change
	nm		$\text{cm}^{-1} \mu\text{M}^{-1}$	μM	%
WT	420	390	0.048 (0.002)	8.3 (0.6)	38
I120W	422	389	0.050 (0.000)	3.5 (0.3)	39
T309F	426	389	0.042 (0.002)	16.4 (3.0)	33
I369W	427	390	0.014 (0.000)	18.0 (4.1)	11

smaller amino acid caused preferential formation of 4-OH MDZ as judged by at least a 2-fold decrease in the relative ratio of 1'-OH/4-OH MDZ formation. Furthermore, the substitution of residues Phe-108, Ile-120, Ile-301, Phe-304, or Thr-309 with a larger amino acid favors 1'-OH MDZ formation. Noticeably, residues Ile-120, Phe-304, and Thr-309 form part of either subgroup, depending on the actual substitution made. Whereas the substitution of these residues with a smaller amino acid caused preferential formation of 4-OH MDZ, the substitution of the same residues with a larger amino acid favors 1'-OH MDZ formation.

Correlational Analysis. To assess whether the two hydroxylated MDZ products are produced from binding of the substrate in the same or two different pockets, a correlational analysis of 1'-OH and 4-OH MDZ formation by all mutants was performed. It is well known that the mutation of a single residue could selectively increase or decrease the formation rate of either or both metabolites. However, analysis of a large number of residues that span almost the entire putative CYP3A4 active site was expected to show a strong correlation if MDZ were to bind at a single site, because the effect of mutation of a particular residue should be similar on formation of both metabolites in most cases. We also reasoned that if the two metabolites resulted from MDZ binding at two distinct sites, the effect of mutation of various residues would not be the same for both metabolites, and a poor correlation would result. This should be specially valid in the present study, considering that all 15 residues, with the exception of Tyr-307, are thought to directly affect oxidation of a number of CYP3A4 substrates (Khan and Halpert, 2000; Domanski and Halpert, 2001). The correlational analysis of 1'-OH and 4-OH MDZ formation by all mutants showed r^2 values of 0.015 and 0.21 at 25 and 250 μM MDZ, respectively (Fig. 5). These low r^2 values are inconsistent with the existence of a single MDZ binding site in the CYP3A4 active site.

Spectral Binding Studies. A recent study by Hosea et al. (2000) found that the MDZ binding dissociation constant (K_D) is very similar to the K_M value for 1'-OH MDZ formation, indicating that the spectral change caused by enzyme-substrate complex formation is induced mainly by the binding of MDZ in the 1'-OH orientation. To test this, dissociation constants and the maximal absorbance (ΔA_{max}) change due to

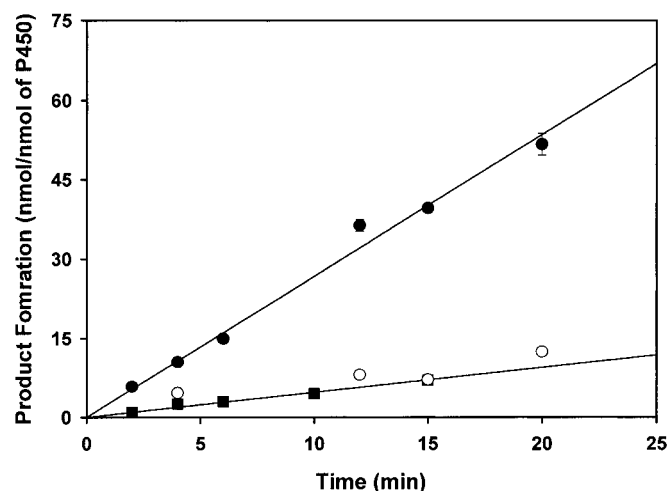


Fig. 7. The time dependence of MDZ hydroxylation by mutant I369W. ■ and ● represent 4-OH MDZ formation at 25 μM and 250 μM MDZ, respectively. No significant formation of 1'-OH MDZ was observed at 25 μM MDZ; at 250 μM , the mutant did show some formation of 1'-OH MDZ (○). The solid lines through the data for 4-OH MDZ formation show a fit to a straight line.

MDZ binding were determined for a few selected mutants and wild-type CYP3A4 (Fig. 6 and Table 3). Interestingly, I120W and T309F, which predominantly form 1'-OH MDZ, show a ΔA_{max} very similar to the wild-type. In contrast, I369W, which mainly forms 4-OH MDZ, shows approximately one-fourth of the ΔA_{max} of the wild-type. Furthermore, although T309F and I369W have very similar dissociation constants, I369W showed only one-third of the ΔA_{max} of T309F. In brief, although these binding studies agree with the suggestion that MDZ binding in the 1'-OH orientation is mainly responsible for the spectral changes upon enzyme-substrate complex formation, MDZ binding in the 4-OH orientation also leads to a small spin-state change in some cases.

The Possible Pathway of Inactivation. One of the most likely causes of CYP3A4 inactivation by MDZ is the irreversible binding of one or more reactive intermediates (Podoll et al., 1995; Schrag and Wienkers, 2001). Thus, to investigate whether one or both of the metabolite pathways leads to CYP3A4 inactivation, three mutants that predominantly form only one of the products were chosen for further analysis. Mutants F304W and T309F mainly form 1'-OH MDZ, while I369W produces 4-OH MDZ preferentially (Table 1). The time dependence of MDZ metabolism by F304W and T309F was very similar to wild-type (data not shown). F304W also showed time-dependent inactivation of progesterone 6 β -hydroxylation by MDZ similar to wild-type with the k_i of 0.15 min^{-1} at 10 μM MDZ (data not shown). The progesterone 6 β -hydroxylation activity of I369W was too low to test for inactivation by MDZ (Domanski et al., 2001). However, the time dependence of MDZ hydroxylation by mutant I369W showed linearity for more than 15 min (Fig. 7). This result indicated that the 1'-OH MDZ and not 4-OH MDZ metabolic pathway is involved in enzyme inactivation.

Discussion

Human CYP3A4 is the most abundant P450 present in both liver and small intestine. Because of the number of

substrates CYP3A4 metabolizes and its proposed involvement in numerous drug-drug interactions, proper evaluation of hepatic as well as intestinal CYP3A4 activity is of paramount importance. Among the various substrates available, MDZ is considered one of the best to probe in vitro and in vivo CYP3A activity (Thummel and Wilkinson, 1998). In the present investigation, we have studied the metabolism of MDZ by CYP3A4 and various SRS mutants. The study provides evidence to indicate that the 1'-OH MDZ metabolic pathway leads to CYP3A4 inactivation. The study also highlights the crucial role of SRS residues in regioselectivity of MDZ hydroxylation and provides information regarding the localization of the proposed multiple MDZ binding sites in the CYP3A4 active site.

Despite the fact that MDZ has been used extensively as a probe of CYP3A4, the inactivation caused by MDZ has not been studied in great detail. Podoll et al. (1995) have shown that MDZ caused a time- and concentration-dependent loss of CYP3A4 catalyzed testosterone 6 β -hydroxylation in human liver microsomes. K_I and k_{inact} were reported to be 16 μ M and 0.32 min⁻¹, respectively, and the inactivation was attributed to the modification of the protein, not heme moiety. More recently Schrag and Wienkers (2001) have shown that MDZ metabolism is prerequisite for CYP3A4 inactivation, and that the loss of enzyme activity is not reversible upon dialysis. These authors also suggested that protein modification rather than heme destruction leads to CYP3A4 inactivation. The present study using recombinant CYP3A4 expressed in *E. coli* showed MDZ causes a time- and concentration-depen-

dent loss of progesterone 6 β -hydroxylation. The $K_I = 5.8 \mu$ M and $k_{inact} = 0.15 \text{ min}^{-1}$ determined from these experiments showed good agreement with those reported (Podoll et al., 1995). We were further able to implicate the 1'-OH metabolic pathway in CYP3A4 inactivation based on several observations. First, the time course of MDZ metabolism by I369W, which predominantly forms 4-OH MDZ, was linear (Fig. 7), in contrast to the nonlinear time course of MDZ metabolism by F304W and T309F, which predominantly formed 1'-OH MDZ. MDZ also caused time-dependent inactivation of F304W similar to the wild-type. Second, there is a good agreement between the K_I determined from inactivation studies of the wild-type enzyme and the K_M for 1'-OH MDZ formation. However, the precise details of the underlying mechanism of enzyme inactivation remain to be established. Binding to the protein of a rearrangement product of the initial 1'-methylene radical, such as a methylene imine (Lanza et al., 1999), is a likely possibility.

Determination of in vitro kinetic parameters is essential for the prediction of drug metabolism by a particular P450 in vivo. However, the exact evaluation of these parameters for CYP3A4 has proven far more difficult than for other P450s because of the unusual substrate kinetics, including activation, autoactivation, partial inhibition, and substrate inhibition often observed (Shou et al., 1994, 2001; Ueng et al., 1997; Harlow and Halpert, 1997, 1998; Domanski et al., 1998, 2000, 2001; Korzekwa et al., 1998). Several hypotheses involving two-site or three-site models as well as the existence of functionally distinct conformers have been proposed to

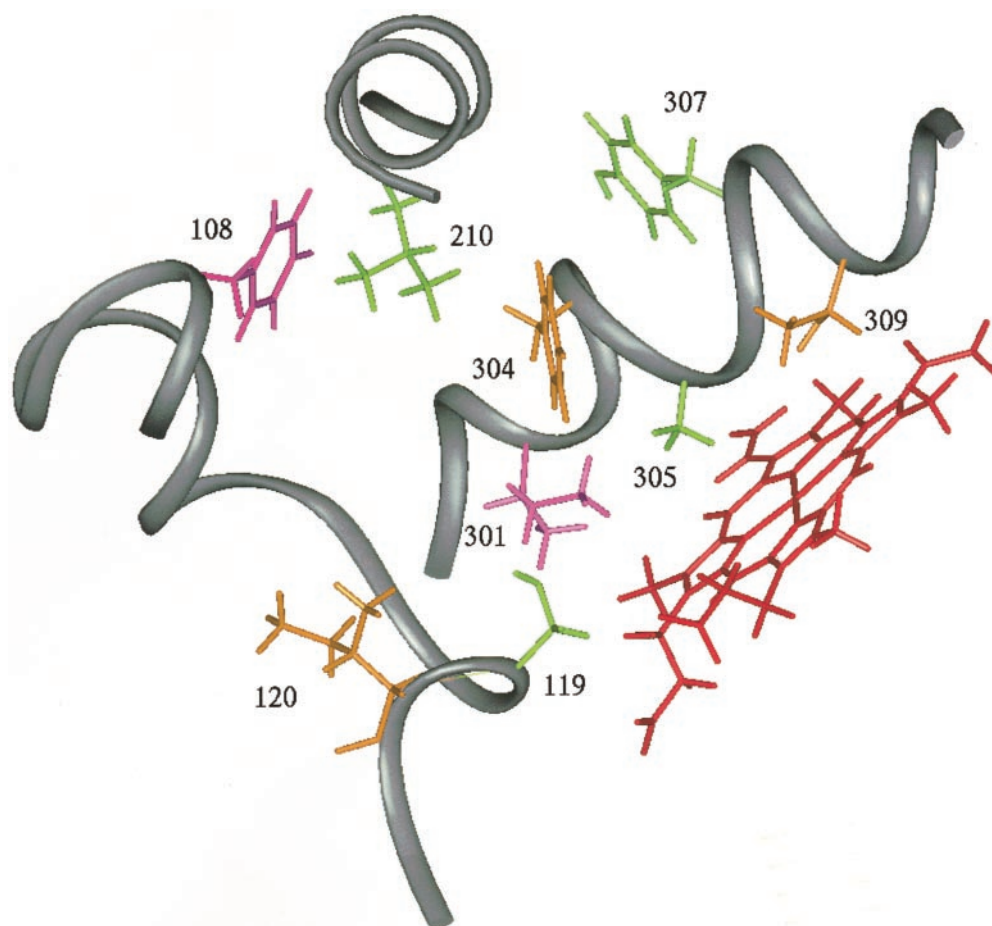


Fig. 8. Molecular model of the CYP3A4 active site showing some of the SRS residues at which side chain substitution had the most significant effect on the regioselectivity of MDZ oxidation. The substitution of residues shown in green with a smaller amino acid caused a decrease in the ratio of 1'-OH/4-OH MDZ formation, whereas the replacement of the residues shown in pink with a larger amino acid caused an increase in this ratio. The residues shown in gold fit well in both categories, showing a decrease or increase in this ratio depending on whether amino acid substitution was smaller or larger, respectively.

explain the unusual CYP3A4 kinetics (Shou et al., 1994, 2001; Ueng et al., 1997; Harlow and Halpert, 1998; Korzekwa et al., 1998; Domanski et al., 2000, 2001; Hosea et al., 2000). Observation of two very distinct K_M values for the two metabolites of MDZ (Ghosal et al., 1996; Hosea et al., 2000), which has been confirmed in our study (Table 1), suggests the existence of two MDZ binding sites in CYP3A4. The differential stimulation/inhibition by ANF and testosterone (Ghosal et al., 1996; Wang et al., 2000) and observation of two distinct K_i values for inhibition of 1'-OH and 4-OH MDZ formation by the peptide YFPF-NH₂ have provided additional evidence to support two binding sites (Hosea et al., 2000).

Two other possibilities to account for the above observations are: 1) two functionally different conformers are responsible for the conversion of MDZ to the different products (Koley et al., 1995), or 2) the substrate binds at a single site but in two different orientations. The fact that an identical nonlinear time course for both metabolic pathways was observed (Fig. 2A), although only the 1'-OH metabolic pathway seems to lead to enzyme inactivation, suggests that the formation of both products is catalyzed by a single conformer of CYP3A4, tending to exclude the first possibility. Correlational analysis provides one way to evaluate the possibility that MDZ binds at only one site but in two orientations. If this possibility were correct, substitution of several SRS residues would be expected to affect the formation of both metabolites in a similar way and a good correlation would be observed. In fact, a reanalysis of the data from previous studies of AFB1 (Wang et al., 1998; Xue et al., 2001) and progesterone (Domanski et al., 2001) oxidation revealed significant positive correlations between the production of two major metabolites by a panel of mutants ($r^2 = 0.51$ between 6 β - versus 16 α -hydroxylation of progesterone, and $r^2 = 0.62$ between 3 α -hydroxylation versus 8,9-epoxidation of AFB1). The strong correlations and similar S_{50} values for the two different products for the both substrates (Ueng et al., 1997; Roussel et al., 2000) are consistent with substrate binding at same site but in two orientations. However, the preponderance of evidence (including the very poor correlations (Fig. 5), two distinct K_M values and the differential effect of various effectors/inhibitors on the two metabolites) is inconsistent with a single MDZ binding site in the CYP3A4 active site.

In recent years, the three common approaches used to understand CYP3A4 active-site dynamics include the use of mathematical models based on analysis of large sets of steady-state kinetic data, the effect of inhibitors or effectors, and site-directed mutagenesis (Domanski et al., 2001). Among these, use of site-directed mutagenesis is the only approach that can provide insight into the structural basis of the functional properties of this enzyme in the absence of a CYP3A4 crystal structure. In fact, in recent years, mutagenesis studies from this and other laboratories have implicated a number of specific amino acids in various CYP3A4 functions, and virtually all the residues that we have proposed to comprise the active site have direct counterparts in available crystal structures (Cupp-Vickery et al., 2000; Williams et al., 2000). In the present study, a closer inspection of the relative rates of formation of 1'-OH and 4-OH MDZ by various mutants also provides a possible way to determine the role of a specific amino acid in regioselectivity of MDZ oxidation and to assess the relative location of the two putative sites (Table

2). This comparison showed that the substitution of residues Ser-119, Ile-120, Leu-210, Phe-304, Ala-305, Tyr-307, and Thr-309 with a smaller amino acid caused preferential formation of 4-OH MDZ (Fig. 8). In contrast, the substitution of residues, Phe-108, Ile-120, Ile-301, Phe-304, and Thr-309 with a larger amino acid favors 1'-OH MDZ formation. The close proximity of several of these residues, as well as the fact that some of them (Ile-120, Phe-304, and Thr-309) form part of both subgroups, indicates that the two putative sites of MDZ may be partially overlapping. The steady-state kinetic analyses of mutants S119A, L210A, F304A, and L373F showed that the relative increase in 4-OH versus 1'-OH MDZ formation was caused by a 4- to 10-fold increase in the K_M value for 1'-OH MDZ, and a 2- to 4-fold increase in the V_{max} for 4-OH MDZ (Fig. 4 and Table 1). Additionally, the increase in the selectivity for 1'-OH MDZ by the mutants I120W and F304W was caused by a 2.5- to 3-fold increase in the V_{max} for 1'-OH MDZ and a slight decrease in the V_{max} for 4-OH MDZ and the K_M value for 1'-OH MDZ. The fact that the alterations in metabolite profile reflected changes in kinetic parameters for the formation of not just one but both metabolites in most cases provides additional support for the close proximity of the two MDZ binding sites. The spectral studies of the mutants showing that MDZ binding in either orientation causes at least a small spin-shift also indicates that a part of CYP3A4 active site may be common for both binding orientations.

In conclusion, the complex kinetic behavior of CYP3A4 still remains one of the most studied yet controversial subjects in P450 research. Although MDZ hydroxylation by CYP3A4 has not been reported to show cooperativity, a number of studies have suggested the possible existence of two MDZ binding sites within the CYP3A4 active site. The present study with MDZ strongly supports this hypothesis and further indicates that the two MDZ binding sites may be in close proximity. The finding that the 1'-OH MDZ metabolic pathway might be causing CYP3A4 inactivation could be of important clinical significance. Because 1'-OH is the only product formed at clinically approved dosage of the drug, the use of MDZ as in vivo probe may require special consideration of CYP3A4 inactivation and the complications that could arise from interaction with flavones and steroids.

Acknowledgments

We would like to thank Dr. Fabienne Roussel for generating I120A and L479A, You Ai He for providing purified mutants, and Dr. Quinmi Wang for providing a molecular model of CYP3A4.

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Address correspondence to: Kishore K. Khan, Ph.D., Department of Pharmacology and Toxicology, The University of Texas Medical Branch, Route 1031, 301 University Boulevard, Galveston, Texas 77555-1031. E-mail: kkkhan@utmb.edu