Chiral Recognition at Cytochrome P450 1A2 Active Site: Effects of Mutations at the Putative Distal Site on the Bindings of Asymmetrical Axial Ligands†

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ABSTRACT: Effects of mutations at the putative distal site of cytochrome P450 1A2 on chiral discrimination for binding (*R*)-(+)- and (*S*)-(−)-1-(1-naphthyl)ethylamine (ligand I), (*R*)-(−)- and (*S*)-(+)-1-cyclohexylethylamine (ligand II), and (*R*)-(+)- and (*S*)-(-)-1-(4-pyridyl)ethanol (ligand III) were studied by optical absorption spectra. The wild-type P450 1A2 exhibited different dissociation constants (K_d) for the *R*- and *S*-enantiomers of these ligands. The R/S ratios of the K_d values for ligands I and II were 5.2 and 2.9, respectively, and the S/R ratio for ligand III was 6.0. Mutations at the putative distal site, such as Glu318Asp and Glu318Ala, remarkably enhanced the discrimination: the R/S ratio of the K_d values for ligand I increased from 5.2 to 20–60, while the R/S ratio for ligand II decreased from 2.9 to 0.8–0.9. These remarkable changes in the R/S ratios were not observed with Glu318Asp mutation for ligand III binding, whereas affinities for both enantiomers of ligand III were markedly decreased by the Glu318Ala mutation. Mutation Thr319Ala increased the R/S ratio of the K_d values for ligand I slightly but markedly decreased the R/S ratio of ligand II (from 2.9 to 0.8) and the S/R ratio of ligand III (from 6.0 to 1.0). Similar enhancements of the chiral discriminations were observed with the mutation Lys250Leu at another putative substrate-recognition site. Differences between the *R*- and *S*-enantiomers of the standard enthalpy and entropy of ligand III binding were changed most remarkably by the Thr319Ser mutation. From these findings, together with other spectral data, it is suggested that (1) Glu318 and Thr319 play important roles in the chiral recognition of asymmetrical axial ligands, (2) Thr319 contributes thermodynamically to the discriminations of those chiral axial ligands, and (3) Lys250 is important in the chiral recognition and may be located close to a ligand access channel and/or a substrate-recognition site of this enzyme.

Eukaryotic cytochrome P450 (P450)[1] is the important heme enzyme which catalyzes monooxidation reactions of exogenous chemicals such as drugs and carcinogens as well as those of important endogenous compounds such as steroids and lipids (Guengerich, 1991; Porter & Coon, 1991; Ortiz de Montellano, 1986). The catalytic activity of eukaryotic P450 shows regioselectivities and also enantiospecificities toward many substrates (Miwa & Lu, 1986; Morris & Richards, 1992; Waxman et al., 1982; Yagi & Jerina, 1982; Yasumori et al., 1991). Chiral recognition or discrimination of substrates in the enzymatic reaction can provide important clues relating to the enzymatic reaction mechanism (Hatano, 1986; Lee & Williams, 1990; Macherey & Lhuguenot, 1990; Petsko, 1992). In the absence of the three-dimensional crystal structure of eukaryotic P450s, it is difficult to understand the origin of the chiral specificity. Nevertheless, by referring to the crystal structure of bacterial P450 101 (Poulos et al., 1985, 1987; Raag et al., 1991) and to alignments of all P450s amino acid sequences (Gotoh, 1992; Nelson & Strobel, 1988), one can infer some of the essential features of the eukaryotic P450 enzyme. Extensive protein engineering studies of bacterial P450 101 (Atkins & Sligar, 1988, 1989; Gerber & Sligar, 1992; Imai et al., 1989; Martinis et al., 1989) and eukaryotic P450s (Furuya et al., 1989; Graham-Lorence et al., 1991; Hanioka et al., 1992; Imai & Nakamura, 1989; Ishigooka et al., 1992; Iwasaki et al., 1991; Zhou et al., 1992) indicate that amino acids at the (putative) distal site significantly contribute to the catalytic activities of this enzyme.

The binding of many external ligands to the heme results in structural changes that can be assessed by monitoring the spin changes associated with the heme (Ortiz de Montellano, 1986). In our previous studies, we made various mutants of microsomal P450 1A2, in which amino acids of the putative distal site, such as Glu318 and Thr319, were changed to other amino acids. We showed how those mutations influence the structure of the heme environment of P450 1A2 in terms of the binding fashion of external axial ligands (Krainev et al., 1991a; Shimizu et al., 1991a,b). However, there has been no spectral study describing how mutations influence the chiral structure of the ligand-binding site of P450 and even of other hemoproteins. In view of the important role of the putative distal site in the binding of external axial ligands and also in the catalytic activities, it is interesting to study how amino acids at the putative distal site determine or influence discrimination of asymmetrical axial ligands and substrates.

In the present study, we show that (1) the wild-type P450 1A2 discriminates between asymmetrical axial ligands with different chiralities and that (2) mutations at the putative distal site of P450 1A2 affect this discrimination. We also examine a Lys250Leu mutant at one of the other putative substrate-recognition sites (SRSs) (Gotoh, 1992; Krainev et al., 1991b, 1992). The mutation Lys250Leu further enhances

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[1] Abbreviations: P450, cytochrome P450 (nomenclature recommended by Nebert et al. (1991)); ligand I, (*R*)-(+)- and (*S*)-(−)-1-(1-naphthyl)-ethylamine; ligand II, (*R*)-(−)- and (*S*)-(+)-1-cyclohexylethylamine; ligand III, (*R*)-(+)- and (*S*)-(−)-1-(4-pyridyl)ethanol; K_d, spectral dissociation constant; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Emulgen 913, poly(oxyethylene) *p*-nonylphenyl ether containing 13.1 oxyethylene units on average; β, isokinetic temperature; $\Delta S°$, standard entropy; $\Delta H°$, standard enthalpy; $\Delta G°$, standard free energy; SRS, substrate-recognition site.

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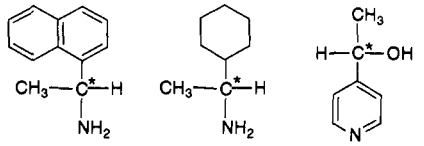

FIGURE 1: Asymmetrical axial ligands used in this study.

the chiral discrimination between the asymmetrical ligands. On the basis of those findings, we discuss the role of amino acids at the putative distal site and a putative substrate-recognition site in the chiral recognition of asymmetrical axial ligands.

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis, DNA sequencing, expression of wild-type and mutant P450 1A2 proteins in *Saccharomyces cerevisiae*, and subsequent purification of P450 1A2 proteins were carried out as previously described (Shimizu et al., 1991b). Approximately 400 nmol (per heme) of P450 1A2 protein was expressed in 1 L of yeast culture under optimum conditions. Yeast microsomes were prepared by crushing yeast cells with an MSK cell homogenizer (B. Braun, FRG) followed by centrifugation at 77000*g* for 90 min. Enzymes were purified by column chromatography on ω-amino-*n*-hexyl-Sepharose and hydroxylapatite (Bio-Rad) after solubilization of microsomes with 0.6% cholic acid (Shimizu et al., 1991b). The wild-type protein was purified essentially as the high-spin form with a Soret peak at 393 nm (Shimizu et al., 1991b). However, mutant proteins contained 10–30% low-spin forms, except for the Glu318Ala mutant which contained more than 70% low-spin form with a Soret peak at 416 nm (Ishigooka et al., 1992; Krainev et al., 1991a). Purified enzymes were prepared in 0.1 M potassium phosphate–20% glycerol (v/v)–1 mM EDTA–1 mM DTT buffer (pH 7.4). For spectral titrations, the buffer containing 0.1% (w/v) Emulgen 913 was used to stabilize the complexes. Concentrations of P450 1A2 enzymes were determined using a molar absorption coefficient of 1.09 $\times 10^5$ M^{-1} cm^{-1} at 393 nm for the high-spin oxidized form and 9.3 $\times 10^4$ M^{-1} cm^{-1} at 447 nm for the difference spectra of the [CO-reduced] – [reduced] form (Shimizu et al., 1991b).

Emulgen 913 was obtained from Kao (Tokyo, Japan). (*R*)-(+)- and (*S*)-(–)-1-(1-naphthyl)ethylamine (ligand I), (*R*)-(–)- and (*S*)-(+)-1-cyclohexylethylamine (ligand II), and (*R*)-(+)- and (*S*)-(–)-1-(4-pyridyl)ethanol (ligand III; Figure 1) were purchased from Fluka Chemie AG (Buchs, Switzerland). Other reagents from Wako Pure Chemicals (Osaka, Japan) were of the highest guaranteed grade and were used without further purification.

Spectral titrations were carried out on a Shimadzu UV-2200 spectrophotometer equipped with a temperature controller (Advantic Thermocool, LCH-4). The time required to finish each spectral transition was about 5 min, depending upon temperature of the solution. To ensure that the temperature of the solution reaches an appropriate temperature, the 1-cm cell was kept in a temperature-controlled holder for 10 min prior to spectrometric measurements. Spectral changes caused by ligands I and III were fitted to the Michaelis–Menten equation under the assumption that a 1:1 ligand–enzyme complex is formed (Krainev et al., 1991a). For the binding of ligand II, Scatchard plots (Fersht, 1985) were used because the binding was composed of two phases. In order to separate two straight lines from the set of experimental points in the Scatchard plot, a regression analysis was performed and lines giving an optimum correlation coefficient were selected. To get standard thermodynamic parameters of ligand bindings, 10–12 separate titration experiments were performed in the temperature range 12–28 °C for each enantiomer in all enzyme preparations. A linear regression analysis using the van't Hoff relation was performed, and 5–7 points giving an optimum correlation coefficient were selected for evaluations of parameters. The values obtained by this procedure differed no more than 10% from the values which were gained by fitting all experimental points. Linear and nonlinear least-square fittings were carried out on an NEC-9801 personal computer as previously described (Krainev et al., 1991a).

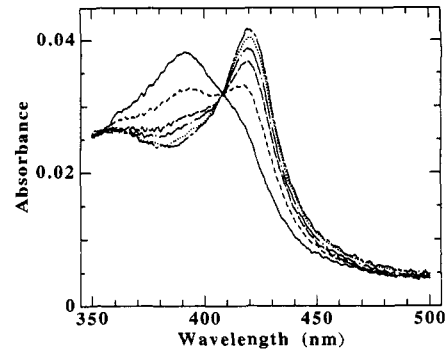

FIGURE 2: Soret spectral changes of the Thr319Ala mutant (0.36 μM) upon addition of 0 (—), 0.13 (- - -), 0.40 (– –), 1.33 (– · –), 6.56 (···), and 12.90 (– – –) mM of *R*-(+)-enantiomer of the ligand III at 24 °C.

Table I: Soret Absorption Peaks (nm) of Low-Spin P450 1A2 Complexes Bound with Asymmetrical Axial Ligands at 25 °C[a]

	ligand I		ligand II		ligand III	
	R^b	S^b	R^b	S^b	R^b	S^b
wild type	420	421	418	418	419	419
Glu318Asp	420	421	413	413	420	419
Glu318Ala	418	418	–[c]	–[c]	–[c]	–[c]
Thr319Ser	421	422	418	418	419	420
Thr319Ala	419	420	419	418	420	421
Lys250Leu	420	421	420	421	420	420

[a] The spectral band widths were 0.5 nm. Experiments to determine the wavelength for each complex were repeated more than six times.[b] *R* and *S* denote *R*- and *S*-enantiomers of the chiral ligand, respectively.[c] A perfect low-spin complex was not formed even by adding a large excess of ligands.

RESULTS

Optical Absorption Spectra. Soret absorption peaks for the wild type and the Glu318Asp, Thr319Ser, and Thr319Ala mutants of P450 1A2 are located at 393 nm indicative of a high-spin complex, while that of Glu318Ala mutant is located at 416 nm, indicating that this mutant contains a low-spin heme (Ishigooka et al., 1992; Krainev et al., 1991a). Adding ligands I and III to the high-spin enzymes results in spectral changes with isosbestic points around 350–355 nm, 408 nm, and 500 nm, giving rise to a peak at 418–422 nm of a nitrogen-bound low-spin complex (Shimizu et al., 1991b) (Figure 2; Table I). Very similar spectral changes were observed for the wild type and Thr319Ser and Thr319Ala mutants by adding ligand II. In contrast, for the Glu318Ala mutant only very small spectral changes were observed upon the addition of ligands II and III, although adding ligand I to the Glu318Ala mutant caused clear spectral changes so as to form a nitrogen-bound low-spin complex. Interestingly, the Soret absorption

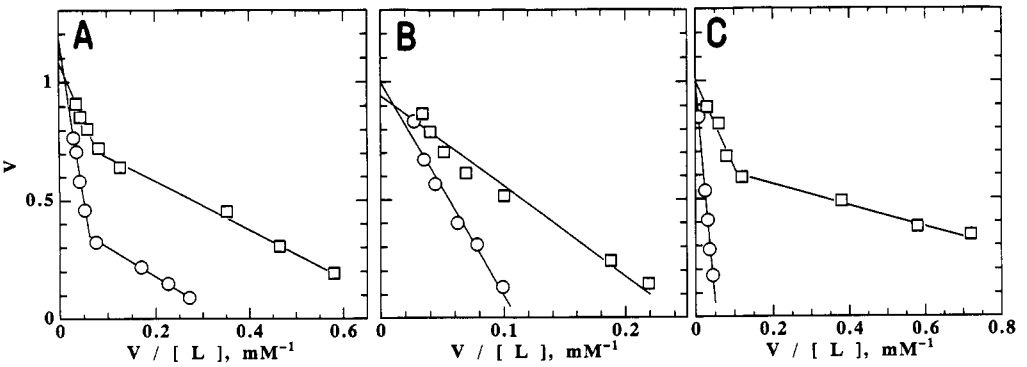

FIGURE 3: Scatchard plots of ligand II binding of (A) the wild type, (B) the Thr319Ser mutant, and (C) the Lys250Leu mutant for the *R*-(−)-enantiomer (○) and the *S*-(+)-enantiomer (□). *V* is the number of moles of ligand bound per mole of the enzyme; [L] is the concentration of free ligand (Fersht, 1985). Spectral titrations were carried out at 25 °C.

Table II: Spectral Dissociation Constants (K_d) of Asymmetrical Ligands I, II, and III from the Wild Type and the Mutants of P450 1A2[a]

	ligand I (μM)		ligand II (mM)				ligand III (mM)	
			1st phase		2nd phase			
	R	*S*	*R* (*n*)[b]	*S* (*n*)[b]	*R* (*n*)[b]	*S* (*n*)[b]	*R*	*S*
wild type	130	26	1.2 (0.43)	1.0 (0.79)	14 (1.18)	4.8 (1.08)	1.1	6.6
Glu318Asp	85	4.2	–	–	6.1	7.1	2.1	8.4
Glu318Ala	230	3.8	–	–	70	84	30[c]	nd[d]
Thr319Ser	300	47	–	–	9.0	3.8	0.16	0.40
Thr319Ala	235	25	–	–	3.0	3.8	0.17	0.17
Lys250Leu	59	3.4		0.45 (0.65)	19	3.7 (1.00)	1.1	15

[a] Data were obtained at 25 °C. K_d values were obtained by averaging parameters of 2–3 separate experiments. In each experiment, data were fitted to straight lines in Scatchard analysis for multiple binding phases or to a Michaelis–Menten type curve for the single binding phase. The values of parameters derived from separate experiments differed by less than 10%. [b] *n* denotes stoichiometry of each binding phase in the case of multiple binding phases. [c] Estimated from very small spectral changes. [d] No spectral change was observed even by adding a large excess of the ligand.

peak at 413 nm of the low-spin Glu318Asp mutant bound with ligand II was located at wavelengths lower than those of other wild-type and mutant complexes, and thus the complex may be an oxygen-bound low-spin complex (Shimizu et al., 1991b) (Table I).

Lys250 is conjectured to be located at one of the putative substrate-recognition sites of eukaryotic P450s and is thought to be remote from the active site (Gotoh, 1992; Krainev et al., 1991b, 1992). The Lys250Leu mutant bound with the three chiral axial ligands formed nitrogen-bound low-spin complexes (Table I).

Differences in the positions of the absorption peak between low-spin complexes bound with the *R*- and *S*-enantiomers were not large (Table I).

Spectral Titrations. The amplitudes of the spectral changes of the wild-type enzyme associated with the binding of ligands I and III obeyed Michaelis–Menten kinetics and suggest that a 1:1 ligand–enzyme complex is formed. Spectral dissociation constants (K_d) obtained in this way are summarized in Table II. In contrast, spectral changes associated with the binding of ligand II to the wild-type enzyme could not be fit utilizing a simple Michaelis–Menten formalism with a single binding phase. These results were analyzed using a Scatchard plot (Figure 3A) (Fersht, 1985). The binding of ligand II was apparently composed of two phases, a high-affinity phase and a low-affinity phase (Figure 3A).

The K_d values of the *R*- and *S*-enantiomers of the three chiral axial ligands for the wild type were clearly different from each other (Table II), suggesting that a binding site(s) of the wild-type P450 1A2 discriminated the chirality of these ligands. The R/S and S/R ratios of the K_d values for these chiral ligands to the wild type were summarized in Table III.

Mutations at the putative distal site of this enzyme both altered the K_d values associated with the binding of all three ligands (Table II) and dramatically affected the chiral selectivity (R/S ratio) of the enzyme for all three chiral ligands (Table III). For example, in the case of Glu318 mutations, the R/S ratio observed for the wild type ($R/S = 5.2$) increased to 20.2 and 60.5 upon substitution of Glu for Asp and Ala, respectively. The same mutations, on the other hand, decreased the R/S ratios for ligand II binding from 2.9 down to 0.8–0.9 (Table III). Another interesting finding is that the two binding phases associated with ligand II binding to the wild-type P450 1A2 disappear upon mutation of Glu318; instead, only a single, low-affinity binding curve is observed for the putative distal mutants (Figure 3B). The Thr319 → Ala mutation decreased the S/R ratio of K_d values for ligand III binding from 6.0 down to 1.0, leading to inability of the Thr319Ala mutant to discriminate between *R*- and *S*-enantiomers of this ligand. However, other mutations did not change the ratio so drastically (Table III).

Table III: R/S and S/R Ratios of K_d Values at 25 °C for Ligands I, II, and III from the Wild Type and Mutants of P450 1A2

	ligand I R/S ratio	ligand II R/S ratio (2nd phase)	ligand III S/R ratio
wild type	5.2	2.9	6.0
Glu318Asp	20.2	0.9	4.0
Glu318Ala	60.5	0.8	–
Thr319Ser	6.4	2.4	2.5
Thr319Ala	9.4	0.8	1.0
Lys250Leu	17.4	5.1	13.7

A surprising result was obtained subsequent to mutation Lys250Leu at one of the putative nonactive site residues (Gotoh, 1992; Krainev et al., 1991b, 1992). We observe that this mutation also enhanced the chiral discrimination of this enzyme (Tables II and III). Namely, the R/S ratios associated with K_d value for ligands I and II and the S/R ratio for ligand III were remarkably enhanced by the Lys250Leu mutation.

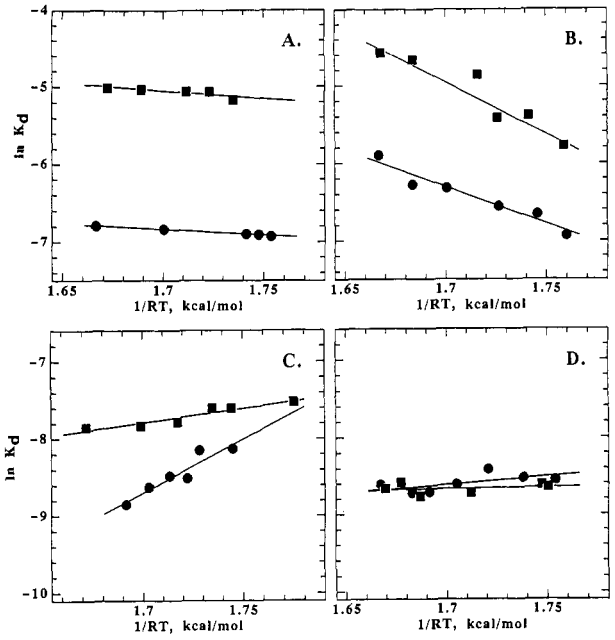

FIGURE 4: van't Hoff plots for the ligand III bindings to (A) the wild type, (B) the Glu318Asp mutant, (C) the Thr319Ser mutant, and (D) the Thr319Ala mutant. Symbols: (●) *R*-(+)-enantiomer; (■) *S*-(−)-enantiomer.

In addition, the two binding phases of the *R*-enantiomer of ligand II became one phase subsequent to this mutation (Figure 3C).

Temperature Dependences. The thermodynamic parameter associated with ligand III binding to the wild-type and mutant P450s 1A2 was examined using van't Hoff plot of the K_d values in order to quantify the chiral discrimination of this ligand (Figure 4). Thermodynamic parameters such as standard enthalpy, $\Delta H°$, standard entropy, $\Delta S°$, and standard free energy, $\Delta G°$, were calculated (Table IV). Differences associated with the binding of the *R*- and *S*-enantiomers of ligand III to the wild-type enzyme are mainly due to $\Delta S°$ values($|R - S| = 5.3$ eu), but the differences in $\Delta H°$ and $\Delta G°$ values are not so large.

The Glu318Asp mutation changed thermodynamic parameters to a certain extent (Figure 4) (Table IV) so that differences ($|R - S|$) between parameters for the *R*- and *S*-enantiomers were changed from 0.5 to 3.4 kcal/mol and from 5.3 to 14.3 eu for $\Delta H°$ and $\Delta S°$, respectively. The Thr319Ser mutation, in contrast, more remarkably enhanced the difference ($|R - S|$) between the *R*- and *S*-enantiomers from 0.5 to 10.2 kcal/mol and from 5.3 to 36.1 eu for $\Delta H°$ and $\Delta S°$ values, respectively. The Thr319Ala mutation did not change $\Delta H°$ values so much as those of the Glu318Asp and Thr319Ser mutations, but the overall effect due to changes of both $\Delta H°$ and $\Delta S°$ values leads to equal $\Delta G°$ values of the *R*- and *S*-enantiomers binding over the whole temperature range studied here (Figure 4D).

The Lys250Leu mutation also changed those thermodynamic values, but the differences between the *R*- and *S*-enantiomers in $\Delta H°$ and $\Delta S°$ values were almost the same as those observed for the Glu318Asp mutant.

When $\Delta H°$ values were plotted against $\Delta S°$ values for the bindings of ligand III to the wild type of four mutants, a linear relationship was obtained with correlation coefficients of 0.999 and 0.995 for the *R*- and *S*-enantiomers, respectively (Figure 5A,B). Thus, enthalpy–entropy compensation effects (Blank & Scheler, 1968; Hsu et al., 1984; Huang & Kimura, 1984; Huang et al., 1986) were observed for both enantiomers. Isokinetic temperatures, β, were calculated at 278 K and 259 K for the *R*- and *S*-enantiomers, respectively, from the slopes of the $\Delta H°$ vs $\Delta S°$ plots (Figure 5). The β values of ligand III binding to P450 1A2 enzymes were comparable to those observed for other hemoprotein reactions (Lamry & Rajender, 1970) (Table V).

DISCUSSION

The active site or ligand binding site of most enzymes is usually asymmetrical, because amino acids constituting the active site of the enzyme are themselves the L-(*S*)-form (Hatano, 1986; Petsko, 1992). Active sites of P450s are also known to be asymmetric, and thus P450s catalyze the transformation of many substrates, showing regio-, stereo-, and enantiospecificities in the hydroxylation reactions (Ortiz de Montellano, 1986; Swanson et al., 1991; Waxman et al., 1982; Yagi & Jerina, 1982; Yasumori et al., 1991). In fact, mutations at substrate-recognition sites of P450 101 successfully changed regiospecificities toward camphor derivatives (Atkins & Sligar, 1988,1989). Theoretical study based on the three-dimensional structure of P450 101 successfully predicted the ratio of the *R*- and *S*-enantiomers of the products (Fruetel et al., 1992; Ortiz de Montellano et al., 1991). Chiral discriminations of P450 enzymes between antagonists and inhibitors have also been discussed (Morris & Richards, 1992; Yoshida & Aoyama, 1990). However, no spectral study showing specifically which amino acids or sites in the enzyme contribute to the chiral recognition to the substrate and/or to external ligand has previously been reported.

In relation to the activation of O_2, the role of the (putative) distal site of P450 101 (Gerber & Sligar, 1992; Imai et al., 1989; Martinis et al., 1989) and microsomal P450 (Ishigooka et al., 1992) has been studied. Furthermore, the distal site of P450 is conjectured to be one of the six substrate-recognition sites (Gotoh, 1992) and thus may also be important in recognition of the substrate. Thr319 (numbered for P450 1A2) in this site is highly conserved for all P450s, while Glu318 is conserved as Glu/Asp for almost all P450s (Nelson & Strobel, 1988). Mutations of these two conserved amino acids at the putative distal site influence binding affinities and binding fashions of external axial ligands to the heme of P450 1A2 (Krainev et al., 1991a; Shimizu et al., 1991a,b). The ligand-access channel of P450s may have a rather hydrophobic character and thus accept rather complicated and larger axial ligands such as metyrapone and phenylimidazole (Dawson et al., 1982; Mims et al., 1983; Shimizu et al., 1991b; White & Coon, 1982) in contrast to hemoglobin and myoglobin which accept rather simple and small axial ligands such as CO, O_2, and some isocyanides [for example, Egeberg et al. (1990), Mims et al. (1983), and Olson et al. (1987)]. Thus, it is interesting to use asymmetrical axial ligands to study asymmetrical character of ligand-binding site and/or the ligand-access channel of P450 and/or to study the role of specific amino acids of P450 in the recognition of chiral molecules, because new valuable structural information could be gained.

Absorption peaks of most of low-spin P450 1A2 complexes bound with ligands I, II, and III are located around 418–421 nm (Table I), suggesting that those complexes for the wild-type and mutant enzymes are nitrogen-bound forms (White & Coon, 1982). However, the absorption peak of the Glu318Asp mutant bound with ligand II was located at 413 nm (Table I), which is indicative of the oxygen-bound low-spin complex (Shimizu et al., 1991b). It is also interesting to note that the Glu318Ala mutation severely hampered the bindings of ligands II and III (Table I). Similar changes in

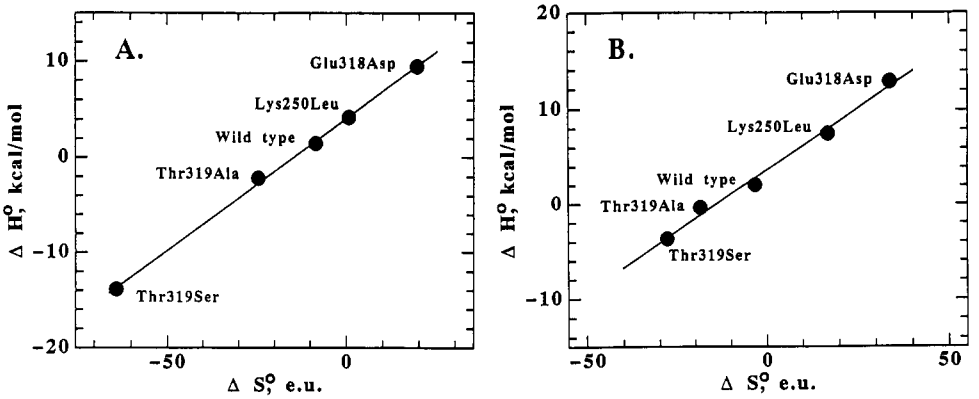

FIGURE 5: Enthalpy–entropy compensations in bindings of the *R*-(+)-enantiomer (A) and the *S*-(−)-enantiomer (B) of ligand III to various P450 1A2 enzymes. Correlation coefficient, $r = 0.999$ (A) and 0.995 (B); Isokinetic temperature, $\beta = 278$ K (A) and 259 K (B).

Table IV: Thermodynamic Parameters of Bindings of (*R*)-(+)- and (*S*)-(−)-Enantiomers of Ligand III to the Wild Type and Mutants of P450 1A2

	$\Delta H°$ (kcal/mol)		$\Delta S°$ (eu)		$\Delta G°$ [a] (kcal/mol)	
	R	*S*	*R*	*S*	*R*	*S*
wild type	1.50 ± 0.10	2.02 ± 0.70	−8.52 ● 0.17	−3.21 ± 1.20	4.04	2.98
Glu318Asp	9.46 ± 1.15	12.9 ± 2.22	19.5 ± 1.96	33.8 ± 3.80	3.65	2.83
Glu318Ala	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]
Thr319Ser	−13.9 ± 2.52	−3.68 ± 0.66	−64.0 ± 4.33	−27.9 ± 1.14	5.17	4.63
Thr319Ala	−2.17 ± 1.24	−0.41 ± 0.93	−24.5 ± 2.12	−18.6 ● 1.59	5.13	5.13
Lys250Leu	4.24 ± 1.74	7.51 ± 1.32	0.70 ± 2.97	16.9 ± 2.28	4.02	2.47

[a] Calculated from $\Delta H°$ and $\Delta S°$ values at 25 °C. [b] Data were not obtained due to very small spectral changes.

Table V: Isokinetic Temperatures (β) Reported from Enthalpy–Entropy Compensation in Various Hemoprotein Reactions

enzyme	reaction	β (K)	ref
P450 1A2	(*R*)-(+)-1-(4-pyridyl)-ethanol binding	278	this work
P450 1A2	(*S*)-(−)-1-(4-pyridyl)-ethanol binding	259	this work
P450 11A1	fluoroaniline binding	408	Sadeque et al. (1988)
horse heart metMb[a]	fluoroaniline binding	571	Sadeque et al. (1988)
P450 11A1	cholesterol binding	403	Hsu et al. (1984)
horse, human, and tubifex metMb[a]	ligand binding	319	Blank and Scheler (1968)
P450s	oxidation–reduction potential	433	Huang et al. (1986)
cytochrome *c*	oxidation–reduction potential	264	Huang and Kimura (1984)

[a] Metmyoglobin.

the binding fashion of 2-phenylimidazole were also observed for the Glu318Asp mutant (Shimizu et al., 1991b). The position of the carboxylate group in the Glu318Asp mutant may be inappropriate to accept these axial ligands.

It is shown in the present study that the ligand-access channel or the ligand binding site of P450 1A2 is chiral and thus discriminates between the *R*- and *S*-enantiomers of chiral ligands (Tables II and III). Mutations at Glu318 and Thr319 altered the K_d values for both *R*- and *S*-enantiomers of all ligands studied (Table II). The discrimination between the *R*- and *S*-enantiomers observed in the wild type was further enhanced by the Glu318 mutations for ligand I binding, while that was decreased by the same mutations for ligand II binding (Table III). It is interesting to note that the two binding phases for ligand II binding were changed to one phase by the Glu318 and Thr319 mutations (Figure 3; Table II). Changes of the R/S ratios for ligands I and II binding to the Thr319 mutants were not so large as compared with the Glu318 mutants. However, the Thr319Ala mutations remarkably decreased the S/R ratio of ligand III binding (Table III). Apparently, amino groups of ligands I and II are located closer to the chiral center than the nitrogen atom of ligand III. Thus, it seems likely that the Glu318 side chain is located closer to the heme in P450 1A2 than that of Thr319. The cyclohexane ring of ligand II may be more flexible than the naphthalene residue of ligand I, leading to a less remarkable effect by mutations. However, the possibility exists that the observed effects are due to local structural rearrangements in the active site. In fact, the crystal structure of the Thr252Ala mutant (Thr252 of P450 101 corresponds to Thr319 of P450 1A2) of P450 101 (Raag et al., 1991) suggest that even such a single point mutation causes distortions in the central portion of the distal helix. This would make the relative position of the ligands in the active site more ambiguous. In addition to the structural contribution of the ligands and the enzyme, the role of the polarity or hydrophobicity of the ligands or environment of the chiral recognition site of the enzyme should be considered.

In contrast to the Thr319 mutations, the Lys250Leu mutation dramatically increases the chiral discrimination for all three axial ligands studied here (Tables II and III). Especially the largest enhancements of the chiral discrimination among the mutants for ligands II and III should be noted. In addition, the enhancement of the chiral discrimination by the Lys250Leu mutation for ligand I was as large as that by the Glu318Asp mutation. Thus, Lys250 seems to be equally important in the chiral discrimination of external axial ligands as has been observed for Glu318 in this enzyme. It was conjectured from optical absorption spectral studies by using bifunctional axial ligands that Lys250 and neighboring ionic amino acids are located at the entrance of the substrate-(or ligand-) access channel of microsomal P450s (Krainev et al., 1991b). In fact, mutations of Lys250 and neighboring ionic amino acids to neutral ones in P450 1A2 enhanced catalytic activities by 3–6-fold toward methoxyresorufin and ethoxyresorufin (Krainev et al., 1992). From the present spectral findings together with the catalytic activities of the Lys250Leu mutant of P450 1A2, it is suggested that Lys250

is certainly located close to a substrate-recognition site and/or a ligand-access channel of this enzyme. It is likely that Lys250 and neighboring ionic amino acids interact with Glu318 or other ionic amino acids at the putative distal site in P450 1A2 in a similar way as in P450 101 where Asp251 is located at the distal site and interacts with Lys178 (Arg186) (Gerber & Sligar, 1992; Krainev et al., 1991a; Poulos et al., 1985, 1987).

A thermodynamic analysis of ligand III binding indicates that the sign of $\Delta H°$ for both enantiomers was changed from plus to minus by the Thr319Ser and Thr319Ala mutations (Table IV). The Thr319Ser and Thr319Ala mutations largely decreased K_d values for ligand III (Table II). Moreover, for the Thr319Ala mutant there is no difference in binding ability of *R*- and *S*-enantiomers of ligand III over the whole temperature range studied (Figure 4D). Thus, Thr319 may contribute more to the binding of ligand III than to those of ligands I and II. Differences in $\Delta H°$ and $\Delta S°$ values between the *R*- and *S*-enantiomers of ligand III were the largest for the Thr319Ser mutant among the P450 1A2 enzymes studied, suggesting that Thr319 is the most important amino acid residue in discriminating between the *R*- and *S*-enantiomers of ligand III. The Glu318Asp mutation changed thermodynamic parameters (Table IV), but those changes are rather small and thus seem less important than those observed in the Thr319Ser mutation. The Lys250Leu mutation also did not seem important thermodynamically in the chiral discrimination for ligand III binding. Therefore, from thermodynamic parameters of the ligand III binding, the important role of Thr319 in the chiral discrimination of ligand III should be emphasized.

Linear relationships were obtained when the values of $\Delta H°$ were plotted against $\Delta S°$ for the *R*- and *S*-enantiomers (Figure 5). The isokinetic temperatures (β) cited in the literature for a number of hemoprotein reactions are summarized in Table V. The β values, 259–278 K, of the bindings of the *R*- and *S*-enantiomers of ligand III were close to those observed for ligand bindings to other hemoproteins (Table V). Since the β values observed in this study were neither zero nor infinity, enthalpy and entropy must compensate each other (Huang et al., 1986). A good enthalpy and entropy relationship for the bindings of the *R*- and *S*-enantiomers of ligand III suggests that single mutations of P450 1A2 described here did not significantly influence the structure of the whole P450 1A2 molecule and that mutants are closely related to each other in the thermodynamic system under study. In fact, circular dichroism spectra of the mutants were essentially the same as that of the wild type (our unpublished results). A participation of the water molecule in the binding reaction may be important from the good enthalpy–entropy compensation relationship (Lumry & Rajender, 1970).

In conclusion, the present work is the first paper demonstrating that absorption spectral changes of a hemoprotein caused by external compounds can provide valuable information about the asymmetrical structure of the ligand-access channel and/or the substrate-recognition site of the enzyme. We have shown that the wild-type P450 1A2 recognizes chiralities of asymmetrical axial ligands and that mutations at putative substrate-recognition sites of this enzyme further enhanced the chiral discrimination of those ligands. We believe that this method will be useful to investigate asymmetrical structure of the substrate- and/or ligand-binding site of the huge molecule such as the enzyme.

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