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Interaction of Fluoroaniline with Cytochrome P-450$_d$ Mutants: Difference Absorption Spectral Studies*

ABU JAFAR MD. SADEQUE, TORU SHIMIZU, KENJI HIRANO, MASAHIRO HATANO

Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, Katahira, Sendai 980, Japan

and YOSHIAKI FUJII-KURIYAMA

Department of Chemistry, Faculty of Science, Tohoku University, Aoba, Sendai 980, Japan

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Abstract

By site-directed mutagenesis near the axial ligand, Cys456, of cytochrome P-450$_d$ (P-450$_d$), we obtained five stable P-450$_d$ mutants, *i.e.* mutant (B), Gly450Ser; mutant (E), Lys453Glu; mutant (G), Arg455Gly; mutant (L), Glu459Ala and mutant (M), Ile460Ser. Bindings of aniline and fluoroaniline to the wild type P-450$_d$ and these five P-450$_d$ mutants were studied with difference absorption spectra at the Soret region. The following results were obtained: (i) binding constants (K_b) of fluoroaniline to wild P-450$_d$ were higher than those to cytochrome P-450$_{scc}$ by more than one order; (ii) K_b value of *para*-fluoroaniline to the wild P-450$_d$ was higher than those of aniline, *ortho*- or *meta*-fluoroaniline by two orders; (iii) K_b values of *para*-fluoroaniline to five mutants were lower than that to the wild type by more than one order; (iv) K_b values of *para*-fluoroaniline to the mutants (E) and (G) were the lowest among those to the five mutants and were lower than that to the wild type by two orders; (v) K_b values of aniline, *ortho*- and *meta*-fluoroaniline to mutant (B) were one order higher than those to the wild and other four mutants. These results indicate that the binding of the external axial ligand to the heme iron of P-450$_d$ is remarkably influenced by mutations at the proximal site possibly due to change in the polarity of the distal site and/or alteration of the secondary protein structure at the distal site.

Introduction

Cytochrome P-450, a class of heme containing enzyme, is a versatile biological catalyst that participates in oxidative transformation of various liphophilic substrates including xenobiotics [1, 2]. Ligand binding studies have been done to understand the active site structure and the mechanism of action of this enzyme [3 and refs. therein]. However, much is yet to be known about the topological feature of the active site as well as the heme environment.

Modern protein engineering provides means of investigating directly the role of specific amino acids in catalytic activity by altering the enzyme structure. Cytochrome P-450$_d$ (P-450$_d$) is predominantly induced by isosafrol in rat liver and has an effective catalytic activity of 2-hydroxylation of 17β-estradiol [4]. Expression of this cytochrome P-450$_d$ in yeast *Saccharomyces cerevisiae* was successful in this laboratory [5], which allowed us to undertake a systematic study for understanding the structure–function relationship of this class of hemoproteins. In succession, we produced a number of cytochrome P-450$_d$ mutants by site-directed mutagenesis, which is the first ever to be reported for the eukaryotic cytochrome P-450 family [6]. Through the mutation study we first experimentally proved that invariant Cys at the carboxy terminal region, Cys456 for P-450$_d$, is the axial ligand of eukaryotic P-450s. It will be very important to know how the binding behavior of external ligands to the heme iron of P-450$_d$ is influenced by mutations near the axial ligand, Cys456.

Aniline is a good ligand for the heme iron as well as a well-known substrate for cytochrome P-450 type enzymes [1–3]. Therefore, as a first step of ligand binding analysis, bindings of aniline and fluorine-substituted aniline compounds to P-450$_d$ mutants were studied. We estimated the binding constants of these ligands to the wild and mutant P-450$_d$s from substrate-induced difference absorption spectra at the Soret region. A comparison of the heme environment of the wild type and mutant P-450$_d$s with P-450$_{scc}$ and metMb was also made in terms of the affinity of aniline and fluoroaniline [3].

*Abbreviations used are: P-450$_d$, cytochrome P-450$_d$; P-450$_{scc}$, cytochrome P-450$_{scc}$; P-420, cytochrome P-420, a denatured form of cytochrome P-450; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Gly450Ser, a mutant in which Gly-450 of wild type P-450$_d$ was changed to Ser. Positions of mutated amino acids in other mutants were abbreviated in the same way.

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Experimental

Mutations of P-450_d by oligonucleotide-directed mutagenesis were done as described previously [6]. Expression vectors of the wild and mutant P-450_d were prepared as described previously [5, 6]. Yeast strain AH22 (a *leu*2 *his*4 *can*1 *cir*$^+$) harboring the expression vector of P-450_d was cultivated as previously reported [5, 6]. The microsomes of the yeast were prepared following the method of Aoyama *et al.* [7] except that the Bead-Beater^{TM} (Biospec Products, U.S.A.) was used for cell crushing instead of a French Press. The prepared microsomes were suspended in 100 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 1 mM DTT at the protein concentration of about 2 mg protein per ml. Microsomes were kept at 4 ℃ and were used within 3 days of preparation. Neither spectral change nor activity change was noticed under these conditions.

Aniline, *ortho*-fluoroaniline, *meta*-fluoroaniline and *para*-fluoroaniline were purchased from Aldrich Chemical Co. All other chemicals were of the highest guaranteed grade and were used without further purification. Protein concentration was determined by the Coomassie Brilliant Blue method [8].

Ligand-induced difference spectra were recorded on a Shimadzu digital UV spectrophotometer (UV-365) equipped with an end-on photomultiplier (HAMAMATSU R-375) at 20 ℃; cuvettes having a 10 mm optical path were used [6].

Results

The yeast harboring the expression vector, pAM82-P-450_d, abundantly expressed rat liver high-spin type P-450_d [5, 6]. From optical absorption spectra of the reduced CO-form of the yeast and yeast microsomes, it was found that the yeast and the microcomes contain P-450_d as a major hemoprotein and contents of non-P-450_d hemoproteins are less than 3% compared with the expressed P-450_d [5, 6]. Thus the spectral changes of the yeast microsomes caused by adding external ligands exclusively reflect those of the expressed P-450_d.

Figure 1A shows typical spectral changes at the Soret region of the expressed P-450_d caused by adding *meta*-fluoroaniline. To obtain the binding constant of the ligand, double reciprocal plots of the spectral change *versus* the concentration of the free ligand were obtained (Fig. 1B). The double reciprocal plots of aniline and fluoroaniline to the wild and mutant P-450_ds clearly showed that the ligand binds to the heme of microsomal P-450_d, forming the 1:1 ligand:heme complex under our experimental conditions. The CO-reduced form of the 1:1 ligand:P-450_d complex did not show a shoulder or a peak at 420 nm, indicating that no denatured form, cytochrome P-420, was formed even after adding excess ligands to the microsomal solution.

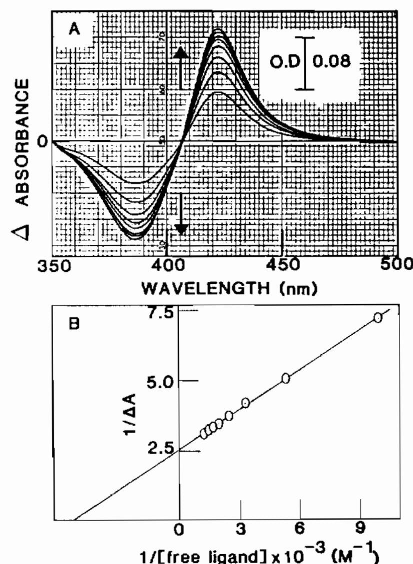

Fig. 1. (A) Spectral changes at the Soret region of microsomal P-450_d caused by adding *meta*-fluoroaniline. The microsomes contained 1.74 μM mutant (B) in a buffer (pH 7.2) consisting of 100 mM potassium phosphate, 1 mM EDTA and 1 mM DTT. Titration was done by adding 1–30 μl of 50 mM *meta*-fluoroaniline solution to 2 ml microsomal solution. (B) Double reciprocal plot of the absorption intensity, $\Delta A_{424\,nm} - \Delta A_{388\,nm}$, *vs.* the concentration of free *meta*-fluoroaniline.

Table I summarizes the binding constants of aniline and fluoroaniline to the wild and mutant P-450_ds. The binding constants of those ligands to P-450_{scc} and metMb are also described for reference. It is noticed that: (i) affinities of aniline or fluoroaniline to the wild P-450_d are apparently higher than those to P-450_{scc} and metMb; (ii) the affinity of *para*-fluoroaniline to wild P-450_d is much higher than those of other ligands by two orders; (iii) affinities of *para*-fluoroaniline to five mutants were lower than that to the wild type by more than one order; (iv) affinities of *para*-fluoroaniline to the mutants (E) and (G) are lower than that to the wild type by two orders; (v) affinities of *para*-fluoroaniline to the mutants (B), (L) and (M) are one-order lower than that to the wild type; (vi) affinities of aniline, *ortho*- and *meta*-fluoroaniline to the mutant (B) are higher than those to the wild and other mutant P-450_ds.

Discussion

From spectral changes of the yeast microsomes harboring the expression vector of P-450_d, it was clearly shown that the difference spectra at the Soret region caused by adding the external ligand are

TABLE I. Binding Constants K_b (M^{-1}) of Aniline Derivatives to Wild and Mutant Cytochrome P-450$_d$ and Other Hemoproteins[a]

Hemo proteins	Aniline	*o*-Fluoroaniline	*m*-Fluoroaniline	*p*-Fluoroaniline	Reference
P-450$_d$ (Wild type)	4.00×10^2	5.66×10^2	8.50×10^2	1.32×10^4	this work
Mutant (B) (Gly450Ser)	6.55×10^3	1.74×10^3	4.70×10^3	3.30×10^3	this work
Mutant (E) (Lys453Glu)	2.41×10^2	2.45×10^2	1.20×10^2	4.00×10^2	this work
Mutant (G) (Arg455Gly)	1.03×10^2	1.60×10^2	2.40×10^2	1.05×10^2	this work
Mutant (L) (Glu459Ala)	4.30×10^2	7.70×10^2	9.31×10^2	2.25×10^3	this work
Mutant (M) (Ile460Ser)	5.50×10^2	6.50×10^2	6.66×10^2	1.46×10^3	this work
P-450$_{scc}$		1.42×10	1.10×10	2.20×10^2	3
metMb		1.40×10	1.05×10	1.80×10	3

[a]Notations of the mutants are the same as those described in ref. 6.

exclusively ascribed to those of expressed P-450$_d$ forming the 1:1 ligand heme complex.

Affinities of fluoroaniline to the wild P-450$_d$ are higher than those to P-450$_{scc}$. The substrate specificity of P-450$_d$ is relatively less restricted in that P-450$_d$ has catalytic activities against several compounds [9]. Thus, the substrate binding site of P-450$_d$ will be more flexible and accessible to several substrates. On the other hand, P-450$_{scc}$ has rigid substrate specificity of only one substrate, cholesterol, suggesting that the substrate binding site of P-450$_{scc}$ may be rather rigid compared to that of P-450$_d$. Thus the difference of the flexibility at the substrate binding site between P-450$_d$ and P-450$_{scc}$ may contribute to the difference of the affinity of fluoroaniline between P-450$_d$ and P-450$_{scc}$.

The affinity of *para*-fluoroaniline to the wild P-450$_d$ is much higher than those of *ortho*- or *meta*-fluoroaniline. A similar trend was observed for the binding of fluoroaniline to P-450$_{scc}$ [3]. The high affinity of *para*-fluoroaniline to the heme iron compared to *ortho*- and *meta*-fluoroaniline may be related with the pK_a value of the fluoroaniline compounds. Thus, the pK_a value of *para*-fluoroaniline is 4.65, while those of *ortho*- and *meta*-fluoroaniline are 3.20 and 3.59, respectively [10]. Since the van der Waals radius of fluorine, 1.4 Å, is very close to that of hydrogen, 1.2 Å [11], stereochemical contribution to the affinity difference among fluoroaniline derivatives to the heme iron may not be important.

Affinities of *para*-fluoroaniline to five mutants were lower than that to the wild type by more than one order. If structural change at the distal site caused by the mutations at the proximal site contributes to this decrease of the affinity, the affinities of aniline and *ortho*- and *meta*-fluoroaniline to the mutants should decrease concomitantly. However, concomitant decrease in the affinity to the mutants was not observed for those compounds. Thus, the decrease of the affinity of *para*-fluoroaniline to the mutants compared with that to the wild type may be related to subtle changes in the polarity of the distal site caused by mutations at the proximal site.

In the mutants (E) and (G), the difference of the affinity of *para*-fluoroaniline from those of aniline, *ortho*- and *meta*-fluoroaniline was not observed. We would speculate that the change of amino acid, Lys453 → Glu for the mutant (E), Arg455 → Gly for the mutant (G), indirectly influenced the charge at the distal site of the heme since alteration of the secondary protein structure will not affect the apparent pK_a value of the *para*-fluoroaniline. For a similar reason, the lower affinities of *para*-fluoroaniline to mutants (B), (L) and (M) compared with that of the wild type may also be caused by a change of polarity at the distal site of P-450$_d$ rather than a structural change at the distal site.

The high affinity of aniline, *ortho*- and *meta*-fluoroaniline to the mutant (B) may also be correlated to the polarity of the distal region of P-450$_d$. If the high affinities of those ligands to the mutant (B) are caused by structural change, the affinity of the *para*-fluoroaniline may increase concomitantly. Since increase of affinity of *para*-fluoroaniline to the mutant (B) was not observed, the change of polarity at the distal site of P-450$_d$ may increase the affinity of aniline, *ortho*- and *meta*-fluoroaniline to the mutant (B).

Therefore, it seems likely that the difference of affinity among aniline derivatives to P-450$_d$ or

among P-450$_d$ mutants are closely correlated to the polarity of the distal region of P-450$_d$. However, it cannot be conclusively ruled out the possibility that alteration of the secondary protein structure at the distal site caused by mutation at the proximal site influences the binding behavior of the aniline derivatives to the mutant P-450$_d$. In addition, the binding behavior of the ligand to P-450$_d$ might also be affected by small differences in the size among the ligand molecules. Further studies to solve this problem are now being carried out in this laboratory.

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