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Remarkable Enhancement of 7-Ethoxycoumarin *O*-Deethylation by Lys250, Arg251 and Lys253 Mutations of Cytochrome P450 1A2

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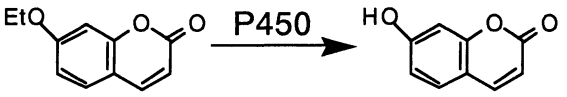
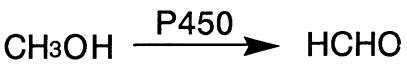
Catalytic efficiency, k_{cat}/K_m, for 7-ethoxycoumarin *O*-deethylation was remarkably enhanced up to 6-fold by mutations at Lys250, Arg251 or Lys253 in cytochrome P450 1A2, while that for methanol hydroxylation was not changed by the same mutations. It is thus suggested that this ionic site is located at or near one of the hydrophobic substrate-interacting sites of this enzyme.

Liver cytochrome P450 1A2 (P450 1A2) is the important heme enzyme with molecular weight 55000 which catalyzes monooxidation reactions of many exogenous compounds such as carcinogens and drugs and endogenous compounds such as steroids and lipids.[1] Site-directed mutagenesis studies have revealed that the putative distal site of P450 1A2 is important in substrate-recognition,[2] O_2 activation,[3] and axial ligand binding.[4] Ionic sites of P450 1A2 are also suggested to be engaged in the recognition of substrates[5] and NADPH-cytochrome P450 reductase.[6] However, it has not been well understood whether or not ionic amino acid(s) of P450 1A2 participates with substrate recognition in terms of K_m or k_{cat}/K_m value. Here we report these kinetic parameters of 7-ethoxycoumarin *O*-deethylation and methanol hydroxylation reactions for





Lys250Leu, Arg251Leu and Lys253Leu mutants of P450 1A2. The parameters were obtained both in the reconstituted system consisting of NADPH and NADPH-cytochrome P450 reductase and in a *tert*-butyl hydroperoxide-supported system. We suggest that the region consisting of the ionic amino acids Lys250, Arg251, and Lys253 may be one of the substrate-recognition sites or may be located at an entrance of a substrate-recognition site for a hydrophobic substrate, 7-ethoxycoumarin, in terms of K_m or k_{cat}/K_m value.

The k_{cat} values for 7-ethoxycoumarin *O*-deethylation reaction obtained in the reconstituted system containing NADPH and NADPH-cytochrome P450 reductase of the Lys250Leu, Arg251Leu and Lys253Leu mutants were a little higher than that of the wild type (Table 1). In contrast, K_m values toward 7-ethoxycoumarin of the mutants were less than a half that of the wild type (Table 1). Catalytic efficiencies, k_{cat}/K_m values, of the mutants were thus more than 2-fold higher than that of the wild type. Especially, the k_{cat}/K_m value of the Arg251Leu mutant was 3.6-fold higher than that of the wild type. Similar enhancements of catalytic efficiency for the 7-ethoxycoumarin *O*-deethylation reaction by the mutations were observed in the hydroperoxide-supported P450 1A2 reaction in the absence of NADPH and NADPH-cytochrome P450

Table 1. Kinetic Parameters for 7-Ethoxycoumarin *O*-Deethylation Reaction of P450 1A2 Mutants [a)]

P450 1A2	Reductase-supported system [b)]			Hydroperoxide-supported system [c)]		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
	min^{-1}	$10^{-3}M$	$10^3M^{-1}min^{-1}$	min^{-1}	$10^{-3}M$	$10^3M^{-1}min^{-1}$
Wild type	0.75	1.41	0.53	0.95	0.60	1.58
Lys250Leu	0.79	0.78	1.01	0.85	0.21	4.05
Arg251Leu	1.07	0.56	1.91	1.24	0.13	9.54
Lys253Leu	1.05	0.60	1.75	1.23	0.20	6.15

a)Site-directed mutageneses, expressions in *Saccharomyces cerevisiae* and purifications of the wild-type and mutant proteins were carried out as described.[5,6)] Parameters were obtained as previously described.[2,3,6)] Experimental errors were less than 10%. b)Parameters were obtained in the reconstituted system consisting of NADPH and NADPH-cytochrome P450 reductase at 25 °C. c)Parameters were obtained in the system containing *tert*-butyl hydroperoxide at 25 °C.

Table 2. Kinetic Parameters for Methanol Hydroxylation Reaction of P450 1A2 Mutants [a)]

P450 1A2	Reductase-supported system [b)]			Hydroperoxide-supported system [c)]		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
	min^{-1}	M	$M^{-1}min^{-1}$	min^{-1}	M	$M^{-1}min^{-1}$
Wild type	8.6	1.3	6.6	4.9	5.6	0.9
Lys250Leu	11.2	1.8	6.2	1.8	1.3	1.4
Arg251Leu	5.7	0.8	7.1	1.6	1.3	1.2
Lys253Leu	10.3	1.9	5.4	3.4	4.3	0.8

a)Parameters were obtained as previously described.[2,3,6)] Experimental errors were less than 20%. b)Parameters were obtained in the reconstituted system consisting of NADPH and NADPH-cytochrome P450 reductase at 25 °C. c)Parameters were obtained in the system containing *tert*-butyl hydroperoxide at 25 °C.

reductase (Table 1). Namely, catalytic efficiencies, k_{cat}/K_m values, of the mutants were much higher than that of the wild type. Here again, the k_{cat}/K_m value of the Arg251Leu mutant was 6-fold higher than that of the wild type.

We obtained the same kinetic parameters for the methanol hydroxylation reaction of the mutants both in the reductase-supported reconstituted system and the hydroperoxide-supported system (Table 2). Catalytic

efficiencies, k_{cat}/K_m values, of the mutants toward methanol hydroxylation were not much different from that of the wild type in the reconstituted system. In the hydroperoxide-supported system, the k_{cat}/K_m values of the mutants were also not much different from that of the wild type.

The crystal structure of bacterial P450 101[8] and sequence alignments of P450s[9] are helpful to understand the structure-function relationship of eukaryotic P450s. The ionic region consisting of Lys250, Arg251 and Lys253 is conjectured to be located near the N-terminal end of the G-helix in the three-dimentional structure of P450 101.[8] It was suggested[10] that these conserved basic amino acids are important for electrostatic interactions with some axial ligands having a diphosphoric acid residue, thus it was implied that they are located near the entrance of the axial ligand access channel. In fact, *iso*propanol binding affinity to the Arg251Leu mutant was 3.7-fold higher than that to the wild type (our unpublished result). The present results indicate that kinetic parameters toward 7-ethoxycoumarin *O*-deethylation reaction were decidedly changed by the mutations. Especially it is noted that the K_m values for 7-ethoxycoumarin were remarkably decreased by the ionic mutations in both electron-transfer systems, perhaps owing to the increased hydrophobicity caused by the mutations. The kinetic parameters for 7-ethoxycoumarin *O*-deethylation by the ionic mutants in the reductase-supported system certainly accord with those in the hydroperoxide-supported system (Table 1). Kinetic parameters of catalytic functions of the putative distal mutants of P450 1A2 are different between the reductase- and hydroperoxide-supported systems.[7] Thus roles of the heme distal amino acids in the catalytic function of P450 1A2 appear to be different between the reductase- and hydroperoxide-supported systems in terms of the kinetic parameters.[6,7] However, the hydroperoxide-supported system is used to examine whether a mutated site is a substrate-binding site or not,[11] because the kinetic parameters of P450 mutants at putative substrate-recognition sites in the reductase-supported system quite correspond to those in the hydroperoxide-supported system. Thus, it is suggested here that the ionic site is located apart from the heme active site so that the ionic mutations do not influence the heme active site and that the ionic site is located at or near one of substrate-binding sites of this enzyme. The alkyl part of the *tert*-butyl hydroperoxide molecule will partially interact with substrate-binding site(s) of the enzyme, which may cause structural change(s) near the ionic site. This may be a part of reasons why K_m values of the 7-ethoxycoumarin *O*-deethylation in the hydroperoxide-supported system are as a whole lower than those in the reductase-supported system (Table 1). The methanol hydroxylation is partly caused by H_2O_2, which is produced by the reductase itself.[3,6] Thus the k_{cat} values of the methanol hydroxylation may in part reflect the affinity for and/or reactivity toward H_2O_2 of the mutant enzymes. Effects of the mutations on the K_m values of the methanol hydroxylation are different from those of the 7-ethoxycoumarin *O*-deethylation in both systems. Perhaps the substrate-binding site(s) and/or the substrate-access channel of the enzyme is not exactly the same for the two substrates.

In conclusion, we suggest here that the ionic region consisting of Lys250, Arg251 and Lys253, especially Arg251, is located at or near the hydrophobic substrate-recognition site of P450 1A2 in terms of K_m and k_{cat}/K_m values. A recent report indicates that the structure of substrates-binding site(s) of P450 BM3 is different from that of P450 101.[12] It seems likely, however, that at least the structure of this ionic region of P450 1A2 is similar, if not the same, to that of P450 101.

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