CHEMISTRY LETTERS, pp. 973-976, 1991.

Remarkable Changes in Catalytic Activity toward Testosterone of Engineered Cytochrome P-450$_d$ by Mutations at Putative Distal Site

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The catalytic activity, 6β-hydroxylation, toward testosterone of engineered cytochrome P-450$_d$ was three-times increased by Thr319Ala mutation at the putative distal site, while that was abolished by mutations Ala315Ser and Thr322Ala at the same site, suggesting the important role of this region in catalytic activity toward the steroid of cytochrome P-450$_d$.

A family of heme-containing proteins, cytochrome P-450 (P-450), catalyzes the oxidation of a wide variety of the endogeneous compounds (e.g., steroids, lipids, and prostaglandins) and xenobiotics (e.g. drugs and carcinogens).[1] Although three-dimensional structure of P-450$_d$ (P-450IA2[2]) is not known yet, it is suggested[3] that the whole tertiary structure of this membrane-bound enzyme may be similar to that of water-soluble bacterial P-450$_{cam}$, whose X-ray crystal structure is known.[4]

P-450$_d$ is induced in rat liver microsomes by isosafrole or 3-methylcholanthrene and catalyzes hydroxylation reactions toward several organic compounds.[5-10] Through site-directed mutagenesis, we suggested that the region Asn310 - Thr322 of P-450$_d$ may be located at the distal region of the heme in terms of spectroscopic studies[11] and catalytic activities.[12] In the present paper, we report that the catalytic activity, 6β-hydroxylation of testosterone, of engineered P-450$_d$ was remarkably changed by the mutations of this region. Especially Thr319Ala mutation increased three-times the catalytic activity of this enzyme.

Table 1. 6β-Hydroxylation Activities of Wild Type and Mutant P-450$_d$s toward Testosterone

Mutants	Time[a]	Turnover number[b]	Turnover number ratio against the wild type
	min	nmol·min^{-1}·(P-450 nmol)$^{-1}$	
Wild Type	1	0.113	-
Asn310Leu	1	0.158	1.4
Ile312Leu	5	0.079	0.7
Phe313Tyr	5	0.068	0.6
Ala315Ser	5	0	0
Glu318Asp	5	0.068	0.6
Thr319Ala	1	0.325	2.9
Thr322Ala	5	0.034	0.3
Phe325Ser	5	0.136	1.2
Phe325Thr	5	0.090	0.8

a)For mutants having low activities, longer times were necessary to obtaine correct activities.

b)Expression of rat liver P-450$_d$ in yeast and preparations of yeast microsomes of the wild type and mutant P-450$_d$s were described previously.[11,12] NADPH-cytochrome P-450 reductase was purified as described previously.[14] The reaction products were analyzed and quantitated by a reverse phase C18 column (TOSOH TSKgel ODS-80T$_M$ 250x4.6 mm inner diameter) on a Waters 600E multisolvent delivery system with progesterone as an internal standard. The reaction products were eluted with a concave gradient of 50% methanol - 49% water - 1% acetonitrile to 85% methanol - 13% water - 2% acetonitrile for 40 min followed by isocratically for an additional 20 min at a flow rate of 0.8 ml/min. All HPLC analyses were performed at ambient temperature and the metabolites were monitored at 254 nm. The enzymatic reaction in yeast microsomes were carried out in a 500 μl solution consisting of 0.2-1.0 nmol P-450$_d$, 200 units of NADPH-cytochrome P-450 reductase, 250 nmol of testosterone, and 0.1 M potassium phosphate buffer(pH 7.0). After the mixture was incubated for 1 min at 37 °C, the reaction was started by adding 500 nmol of NADPH. The mixture was incubated for another 1 min or 5 min at 37 °C and was terminated by adding 500 μl of cold ethyl acetate. The aqueous phase was extracted twice with ethyl acetate and the combined organic solution was concentrated in vacuo. The sample for HPLC was prepared by adding 100 μl of methanol to the residue. The portion, 10 μl, of the methanol solution was subjected to HPLC analysis after filtration.

Yeast microsomes harboring an expression vector of the wild type P-450_d showed 6β-hydroxylation activity toward testosterone (Table 1). The 6β-hydroxylation activity observed for the engineered wild type P-450_d was smaller than that observed for native rat liver P-450_d.[5-10] These activities observed for microsomes harboring the wild type cDNA were not detected for microsomes harboring a shuttle vector, PAM82, lacking P-450_d cDNA.

Distal site of P-450 is very important for activation of molecular oxygen and binding of the substrate for the hydroxylation reaction to occur. In our previous report, it was suggested that the region from Asn310 to Phe325 of membrane-bound P-450_d must be located at the distal site of the heme,[11] which is in accordance with suggestions raised by X-ray crystal structure of water-soluble P-450_{cam}[4] and alignments of amino acid sequence of P-450s.[3] We here obtained catalytic activity of nine putative distal mutants, Asn310Leu, Ile312Leu, Phe313Tyr, Ala315Ser, Glu318Asp, Thr319Ala, Thr322Ala, Phe325Ser, and Phe325Thr, toward testosterone (Table 1). The turnover number of the mutant Thr319Ala was three times higher than that of the wild type, while those of the mutants Ala315Ser and Thr322Ala were negligible compared with that of the wild type.

In our previous papers, we suggested that amino acids at the putative distal site of P-450_d are very important for catalytic activities toward some xenobiotics such as acetanilide, benzphetamine, and 7-ethoxycoumarin in relation with the flexibility of the substrate molecule.[12] Present findings indicate that at least Ala315, Thr319, and Thr322 at the putative distal site are very important for catalytic activity toward testosterone. In particular, it is interesting to note that Thr319Ala mutation enhanced the activity toward testosterone. Ala315 is well conserved as Ala or Gly for all P-450s. Thr319 is well conserved for all P-450s, while Thr322 is conserved for microsomal P-450s.[3] Thus, it is imagined that those three amino acids will be very important for catalytic activity of P-450s.[11-13] This is the first paper that steroid hydroxylation is remarkably enhanced by the mutations at the putative distal site of membrane-bound microsomal P-450. One cannot rule out the possibility that activities of microsomal engineered P-450_d are different from those of the reconstituted system. Further studies for purified P-450_d mutants are in progress in our laboratory.

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(Received March 20, 1991)